FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)
1059.00051	09/914277

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/04798	February 24, 2000	February 24, 1999

TITLE OF INVENTION

ANTI-ANGIOGENIC KRINGLE PROTEIN AND ITS MUTANTS

APPLICANT(S) FOR DO/EO/US

Dexian Dou, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail EL901882301US
23. ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/914277	PCT/US00/04798	1059.00051

24. The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$1,000.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	4 - 20 =	0	x $18.00	**$0.00**	
Independent claims	3 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,000.00**	
☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$500.00**	
SUBTOTAL =				**$500.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$500.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$500.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$500.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **11-1449** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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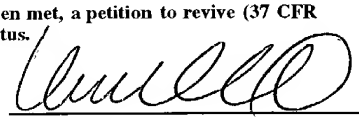

SIGNATURE

Kenneth I. Kohn

NAME

30, 955

REGISTRATION NUMBER

August 24, 2001

DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application: Dexian Dou, et al.

National Phase of: PCT/US00/04798

International Filing Date: 24 February 2000

For: ANTI-ANGIOGENIC KRINGLE PROTEIN AND ITS MUTANT

Attorney Docket Number: 1059.00051

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231
Box PCT

Dear Sir:

Please preliminarily amend the above-captioned patent application prior to examination on the merits, consistent with the instructions found attached hereto:

IN THE SPECIFICATION:

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application is a National Phase Concerning a Filing Under 35 U.S.C. 371, claiming the benefit of priority of PCT/US00/04798, filed 24 February 2000, which claims the benefit of priority of United States Provisional Application Serial Nos. 60/121,341, filed 24 February 1999; 60/121,633, filed 25 February 1999; and 60/166,176, filed 18 November 1999, all of which are incorporated herein by reference.

REMARKS

The above amendments add no new matter and are merely made to more accurately describe and claim the invention, to claim benefit of priority, and to eliminate multiple claim dependencies.

Respectfully solicited,

Kohn & Associates

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Dated: August 24, 2001

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee", EL901882301US, service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231, Box PCT.

Angel Webb

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Page 1, after the Title, please insert the following section:

--CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application is a National Phase Concerning a Filing Under 35 U.S.C. 371, claiming the benefit of priority of PCT/US00/04798, filed 24 February 2000, which claims the benefit of priority of United States Provisional Application Serial Nos. 60/121,341, filed 24 February 1999; 60/121,633, filed 25 February 1999; and 60/166,176, filed 18 November 1999, all of which are incorporated herein by reference.--

AN ANTI-ANGIOGENIC KRINGLE PROTEIN AND ITS MUTANTS

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention relates to the use of kringle proteins for the treatment of disease. More specifically, the present invention relates to the use of kringle proteins for the treatment of cancerous lesions.

2. DESCRIPTION OF RELATED ART

Normal physiological growth of new blood vessels in the mature organism is tightly delimited in time and restricted in localization. It occurs during wound repair, embryogenesis, corpus luteum formation, and the menstrual cycle (Folkman et al., 1996; Folkman, 1995). In contrast, nonphysiologic neovascularization is found in many disorders such as chronic inflammation, psoriasis, diabetic retinopathy, and cancer. Several avenues of experimental evidence indicate that neoangiogenesis is a critical and necessary step in the metastatic process both for tumor survival and progression. The development of a network of blood vessels is a prerequisite for the local expansion of tumor colonies beyond the size (0.125 mm2) restricted by oxygen and nutrient diffusion (Liotta et al., 1991; Folkman et al.,1992).

Recent data from transgenic mouse models of tumorigenesis indicate that the induction of angiogenesis appears during early stages of tumor development, suggesting that this event represents a potentially rate-limiting step (Hanahan et al., 1996). Folkman (Folkman, 1971) first hypothesized the utility of anti- angiogenic therapies, and Liotta and colleagues (Liotta et al.,

1974) first demonstrated the relationship of neovascularization to metastatic progression in melanoma where tumors without signs of new vessel development rarely metastasized. Neovascular development has been proven to be a marker for invasive potential and is diagnostic of the most aggressive primary brain cancer, glioblastoma multiforme (Mikkelsen et al., 1995). Immunohistochemical staining for Yon Willebrand's factor, factor VIII or CD34 has been used to identify tumor-related microvessels. Numeric assessment of tumor vascularity has been shown to have a negative prognostic significance in a variety of human solid tumors including breast cancer, prostate cancer, ovarian cancer, and in primary brain tumors. Thus, neoangiogenesis is a novel biological target in tumors since angiogenesis involves local endothelial cell invasion, proliferation, and migration in response to many of the same cytokines frequently produced by tumors.

The organization of proliferative neovessels in malignant tumors appears to be the result of a complex pattern of expression of a number of growth factors and their signaling pathways. In a fashion analogous to the positive-proliferative oncogenes being balanced by the growth inhibitory tumor suppressor genes, angiogenesis has been described as being regulated by angio-promoting factors (bFGF, VEGF, PDGF, TGF-β) and angio-inhibitors (TSP-I, GDAIF). Angio-inhibitors in glioma cells appear to be subject to regulation by p53 or genetic material on chromosome 10. The physiologic invasion required for neovascularization, local basement membrane remodeling capillary bud formation followed by vascular tube formation, has been shown to involve the same steps as tumor invasion (Kohn et al., 1995, Liotta et al., 1991). The balance between the martix metalloproteases (MMPs) and their inhibitors in the local environment results in local proteolysis or an inhibition of invasion. Endothelial cells have been shown to produce gelatinase A and to be sensitive to autocrine and paracrine production of tissue inhibitors of metalloproteinases (TIMPs) 1 and 2 and cathepsin B (Mikkelsen et al., 1995). Hypoxic cancer cells in the core of

tumors secrete a number of growth factors such as VEGF (vascular endothelial growth factor), IL-8 (interleukin-8), HGF (hepatocyte growth factor) and bFGF (basic fibroblast growth factor) that stimulate proliferation of endothelial cells and cause rapid sprouting of new capillaries from adjacent blood vessels (White et al., 1995). The $\alpha_v\beta_3$ integrin (Hruska et al., 1995), mediates a significant parallel pathway in angiogenesis, and also has been found to be highly expressed on angiogenic blood vessels.

Recent progress in the understanding of a paradoxical clinical observation has been made, based on the phenomenon that removal of a primary tumor increases the growth rate of remote metastatic tumor (Fisher et al., 1983; Fisher et al., 1989; Simpson-Herren et al., 1976). A hypothesis has been proposed to explain this phenomenon (O'Reilly et al., 1993) which purports that a primary tumor stimulates angiogenesis in its own habitat by generating angiogenesis stimulators in excess of angiogenesis inhibitors, and that the circulating inhibitors suppress the progressive growth of latent remote metastases. Once the primary tumor is removed, the source of circulating inhibitor is eliminated and remote metastases are themselves capable of progressive uninhibited growth.

A circulating inhibitor has been identified and named angiostatin. Angiostatin is a 38 kDa internal fragment of plasminogen. It has been demonstrated that human prostate carcinoma cells (PC-3. DU-145, and LN-CaP) express enzymatic activity that converts plasminogen to angiostatin (Gately et al., 1997), suggesting that generation of angiostatin is not an individual event limited to Lewis lung carcinoma.

Increased evidence reveals that generation of angiogenesis inhibitors by primary cancers is widespread. Endostatin. a novel angiogenesis inhibitor of the C-terminal fragment of collagen α1(XVIII) has also recently been

identified from the conditioned media of murine hemangioendothelioma cell line EOMA (O'Reilly et al., 1994). A study of human blood ultrafiltrate (hemofiltrate, HF) from patients with chronic renal insufficiency found that endostatin is present in the human blood circulation (Standker et al., 1997).

Endostatin is a 20 kDa protein. Both wild-type and recombinant murine endostatin at a concentration of approximately 50 nM, completely inhibit the proliferation of bovine capillary endothelial cells. However, the purified human endostatin from hemofiltrate (HF) failed to inhibit the proliferation of bovine endothelial cells at the same concentration. Alignment of human circulating endostatin with murine endostatin isolated from mouse hemangioenqothelioma cell medium, indicates that the N-terminus of circulating human endostatin is 12 amino acids shorter than the mouse endostatin. There are no reports showing that human endostatin is as active as the mouse one.

Compared with endostatin, angiostatin shows much weaker angiogenesis inhibition activity. A concentration of 50 nM endostatin completely suppressed the bovine endothelial cell proliferation. To obtain the same effect of mouse endostatin, more than 10 times the amount of angiostatin was required. Notable homology has not been found between endostatin and angiostatin at the level of DNA or amino acid sequences. Recently, the plasminogen kringle 5 alone showed inhibition of bovine endothelial cell proliferation at the same concentration as that of angiostatin which is composed of kringle 1 to 4 domains. Additional experiments indicate that individual kringles in angiostatin have varied anti-angiogenesis activities. These data suggest that the kringle structure is essential for expression of the anti-angiogenesis activity of angiostatin. Each kringle possessing anti-angiogenesis activity can act as a "mini-angiostatin".

There are no kringle-like structures present in the endostatin molecule. The anti- angiogenic mechanisms of endostatin appears completely different from angiostatin. It is important to know the anti- angiogenic activity of a single molecule containing the two complete different structures of kringle 5 and endostain. It would therefore be useful to determine whether the fusion of human plasminogen kringle 5, the mini-angiostatin, with the human endostatin, the strongest angiogenesis inhibitor so far discovered, would create a potentially potent angiogenesis inhibitor "KED". The estimated MW of "KED" is 30,001 Da, less than the 38 kDa of angiostatin.

Human tissue plasminogen activator (tPA) is a thrombolytic agent used primarily to treat myocardial infarction (Topol et al., 1988; Stump et al., 1989), ischemic stroke (National Institute, 1997; Wardlaw et al., 1997) and pulmonary embolism (Goldhaber et al., 1988; Parker et al., 1988). tPA has a modular structure, consisting of a fibronectin-like "finger", an epidermal growth factor domain, two kringle structures and a serine protease domain (Pennica et al., 1983; Patthy, 1985). The primary structure of kringles contains approximately 90 amino acids, with a rigidly conserved triple disulphide bond pattern. The two tPA kringles show a high homology to each other sharing 51% identical amino acid residues. The tPA kringles also are markedly homologous with the kringles of plasminogen. There are 28 to 35% identical residues between two tPA kringles and the five kringles in plasminogen (Fig 1).

Kringles are involved in basic protein-protein interactions. There are two known major biological functions of kringles, fibrinogenosis and angiogenesis. Kringles in tPA and plasminogen provide the site-specific thrombolytic reaction in the fibrinogenosis system (de Munk et al., 1989; Nesheim et al., 1990). The lysine-binding site in tPA or plasminogen kringles, which can interact with internal lysine in fibrin is responsible for fibrin binding (Horrevoets et al., 1994; Urano et al., 1991). Kringle structures of

plasminogen have been demonstrated to be the key player of angiostatin. in the natural anti-angiogenesis reaction chain (Cao et al., 1996). However, the site(s) for expression of anti-angiogenesis activity of kringles are unknown. Also unknown is whether tPA kringles have the same anti-angiogenic activity as plasminogen kringles.

Binding of a kringle to endothelial cells can be an indispensable condition for anti- angiogenic activity. Studies of the interaction between tPA and endothelial cells showed that tPA binds to endothelial cells in a kringle (tPA kringle 2)-mediated manner (Mulder et al., 1997; Cheng et al., 1955), providing indirect evidence that tPA kringle 2 binds with endothelial cells. However, neither the lysine-binding site nor the ability of binding with endothelial cells has been found in the tPA kringle domain. Properties of tPA kringle 2, together with high homology between tPA and plasminogen kringle suggest that the tPA kringle 2 can posses anti-angiogenesis activity, which lead the present inventors to investigate the anti-angiogenesis function of tPA kringles.

It would therefore be useful to establish that distinct active sites in kringles play different roles in fibrinogenosis and angiogenesis. Additionally, it would be useful to show that kringles lacking lysine-binding site show anti-proliteration activity of endothelial cells. indicating that the lysine-binding site in kringles is not the site for anti-angiogenic function.

It would therefore be useful to clone human endostatin with the 12 amino acid at the N-terminus and to indicate that the recombinant human endostatin is as active as its mouse counterpart. It is thus set forth by the present application that the deletion of 12 amino acids from the circulating human endostatin is the cause of enzymatic degradation.

Since abnormal growth of blood vessels is associated with a number of

diseases such as cancer, diabetes. arthritis, angiosarcoma and angiokeratoma, it would be useful to identify agents inhibiting growth of new blood vessels which represents a novel treatment of these diseases. The tPA kringle 2 peptide is a small protein of 9.5 kDa. Therefore, the modified version with mutation of histidine to tyrosine shows strong anti-angiogenic activity which is useful for a pharmaceutical composition.

SUMMARY OF THE INVENTION

According to the present invention, there is provided a pharmaceutical composition including at least one kringle protein and a pharmaceutically acceptable carrier. Also provided by the present invention is a method of treating a disease by administering an effective amount of a compound from the group consisting of kringle proteins, KED and a kringle derived from the tPA protein.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows a schematic representation of molecular cloning and fusion of KED gene; a vector pEXdna Y containing T7 promoter and dnaY gene having been constructed for KED gene expression; KED gene generated by fusion of plasminogen kringle 5 cDNA and collagen α1(XVIII) fragment being shown; plasminogen kringle 5 cDNA being derived from human liver mRNA; collagen αl (XVIII) cDNA being from human kidney;

Figure 2 is a photograph showing the expression of the KED protein; the SDS-PAGE shows the induction of KED expression by 0.5 mM IPTG in 3 hours, shown as a Coomassie Blue-stained 10% polyacryl amide gel with 50 *ug* proteins on each lane;

Figure 3 is a photograph showing the isolation and purification of the KED protein; pure KED protein (2.5 *μg)* was analyzed by 10% SDS-PAGE with Coomassie Blue-staining which reveals a single KED peptide migrated at the position of 30 kDa;

Figures 4 A and B are graphs showing the *in vitro* anti-angiogenic activity of purified KED; A. Cells on each assay filter were counted; B. KED activity was analyzed in a proliferation assay using bovine endothelial cells; anti-angiogenic activity of KED is compared with endostatin and plasminogen kringle 5;

Figure 5 A and B are photographs showing the inhibition of blood capillaries growth by KED., the *in vivo* anti-angiogenic activity of KED was tested by rat corneal assay; 25 ng bFGF intitiates an aggressive angiogenic response (A) and 25 ng bFGF +12.5 ng KED significantly suppresses (B);

Figure 6 shows the alignment of amino acid sequences of human tPA ($K1_{tPA}$, $K2_{tPA}$) and plasminogen kringles ($K1\text{-}5_{plg}$); the kringle being aligned according to their conserved cysteines (indicated by stars), positions with identical amino acids in all seven kringles are boxed;

Figure 7 is a photograph showing the expression of tPA kringle 2 domain which was denatured in 16% polyacrylamide gel and stained with Coomassie-blue; the tPA kringle 2 domain was purified by an affinity lysine-agarose chromatography;

Figure 8 is a graph showing the test of anti-angiogenic activity of tPA fragments; the proliferation assay used bovine endothelial cells wherein the Asn^{177}-Thr^{63} fragment is tPA kringle 2 domain and Ile^{86}-Thr^{63} contains tPA kringle 1 and 2, also Asn^{177}-Pro^{527} contains tPA kringle 2 and serine protease domain;

Figure 9 is a photograph showing the expression of tPA kringle 2 mutants; Whole cells, equivalent to 50 g proteins, were analyzed by denatured 16% SDS-PAGE;

Figure 10 is a diagram showing the site-directed mutagenesis in tPA kringle 2; mutation are presented by *arrows* with amino acid sequence of tPA kringle 2 domain; mutations in the middle of kringle caused unstable expression; the effects of mutations on anti-angiogenic activity are compared *(internal)*;

Figure 11 is a photograph showing the KED Protein; the KED gene was expressed under a T7 promoter, the recombinant *E. coli* strain BL21 (DE3) bearing the KED gene produced KED protein with an efficiency exceeding 30% of total cellular protein; the KED protein was isolated using French Press at 20.000 psi; the KED inclusion bodies were dissolved by 8M urea and refolded in a redox solution containing 1.1 mM GSH and 0.19 mM GSSG. Refolded KED protein was concentrated by ultrafiltration and was followed by Sephadex 0-25 chromatography and dialysis against PBS buffer; the purified KED protein appeared at the 30 kDa position on the SDS-PAGE;

Figure 12 is a bar graph showing the activity of the KED protein; KED activity was analyzed by a standard proliferation assay using Bovine endothelial cells; cells on each assay filter were counted 5 days after treatment with KED drug; the control indicates endothelial cells proliferation in the presence of bFGF; proliferation inhibition assays were performed at

different concentrations of KED; proliferation (angiogenesis) of endothelial cells induced by bFGF was significantly inhibited by a concentration as low as 8 nM of KED drug;

Figure 13 is a bar graph showing the activity of endostatin (D); endostatin was the strongest angiogenesis inhibitor prior to the construction of the KED molecule, in identical experiments to those of Fig 12 were performed endostatin had no activity at a concentration of 12 nM;

Figure 14 is a graph showing the comparisons of KED with KS and D drug function; angiostatin (Plasminogen Kringle-5) showed weak anti-angiogenesis activity; Endostatin activity is stronger than angiostatin KED drug is the strongest anti-angiogenesis drug;

Figure 15 is a graph showing the animal treatment data, wherein the results are depicted of animals having tumors being treated with either PBS or KED;

Figure 16 is a graph showing the animal treatment data, wherein the results are depicted of another group of animals having tumors being treated with either PBS or KED;

Figure 17 is a graph showing the animal treatment data, wherein the results are depicted of another group of animals having tumors being treated with either PBS or KED;

Figure18 is a photograph showing the expression of tPA kringle 2 mutants; whole cells equivalent to 50 μg protein were analyzed by denatured 16% SDS-PAGE; M is protein, MW is a marker; the S mutant is a tPA k2

Ser^{186} → Lys; the Y mutant is a tPA k2 Tyr^{214} → Phe; the H mutant is a tPA k2 His^{244} → Tyr;

Figures 19 A-C are graphs and photographs showing the anti-angiogenic activity of tPA kringle 2 mutant; tPA kringle 2 H mutant inhibits endothelial cell proliferation (A) in a dose-dependent manner, endothelial migration (B) and corneal angiogenesis (C) where control bFGF pellet (C, left) is inhibited by bFGF +5 ng H mutant (C, right);

Figures 20 A and B are graphs showing the in vitro anti-angiogenic activity of purified KED; the activity was analyzed by proliferation assay (above) and migration (below) using bovine endothelial cells; cells on each assay filter were counted (A); anti-angiogenic activity of KED is compared with that of endostatin (B);

Figures 21 A-C are graphs showing the Hi-Trap Heparin-Sepharose Chromotagraphy results;

Figures 22 A-D are graphs showing cell proliferation and migration upon treatment with KED; (A) shows the absorbance over a six day period; (B) shows the aborbance over a four day period; (C) is a bar graph showing the BAC cell migration when treated with KED; and (D) is a comparative graph showing BAC cell proliferation when treated with angiostatin;

Figures 23 A-C are bar graphs showing comparative data between KED and angiostatin; (A) shows MTT assay for KED; (B) shows U87 cell invasion for angiostatin; (C) shows BAC cell migration for angiostatin;

Figures 24 A and B are a depiction of (A) the pET system cloning sites and (B) the pET vector system circular map and polylinker.

DETAILED DESCRIPTION OF THE INVENTION

Generally, the present invention provides a pharmaceutical composition for the treatment of disease including at least one kringle protein and a pharmaceutically acceptable carrier. Additionally, there is provided a pharmaceutical composition for the treatment of disease including a KED molecule or the kringle from the tPA molecule.

More specifically, the pharmaceutical of the present invention contains therein anti-angiogenic properties, thus enabling the use of the pharmaceutical in treating cancer and other diseases in which anti-angiogenic properties will minimize or alleviate the symptoms of the disease or the disease itself. The present invention includes compositions and methods for the detection and treatment of diseases and processes that are mediated by or associated with angiogenesis. The composition can be isolated from body fluids including, but not limited to, serum, urine and ascites, or synthesized by chemical or biological methods (e.g. cell culture, recombinant gene expression, protein synthesis, and in vitro enzymatic catalysis of plasminogen or plasmin to yield active angiostatin).

Recombinant techniques include gene amplification from DNA sources

using the polymerase chain reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR. The present invention inhibits the growth of blood vessels into tissues such as unvascularized or vascularized tumors.

The present invention also encompasses a composition comprising, a vector containing a DNA sequence encoding for the kringles of the present invention, wherein the vector is capable of expressing the kringles of the present invetion when present in a cell, a composition comprising a cell containing a vector, wherein the vector contains a DNA sequence encoding the kringles of the present invention or fragments or analogs thereof, and wherein the vector is capable of expressing the kringles of the present invention when present in the cell, and a method comprising, implanting into a human or non-human animal a cell containing a vector, wherein the vector contains a DNA sequence encoding the kringles of the present invention, and wherein the vector is capable of expressing the kringles of the present invention when present in the cell. Still further, the present invention encompasses the kringles of the present invention, fragments thereof, antisera, receptor agonists or receptor antagonists that are combined with pharmaceutically acceptable carriers, and optionally sustained-release compounds or compositions, such as biodegradable polymers, to form therapeutic compositions. In particular, the invention includes a composition comprising an antibody that specifically binds to the kringles of the present invention, wherein the antibody does not bind to plasminogen.

Standard molecular biology techniques known in the art and not specifically described are generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989). Additionally, standard methods in immunology known in the art and not specifically described are

generally followed as in Stites et al. (eds), *Basic and Clinical Immunology (8th Edition)*, Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), *Selected Methods in Cellular Immunology*, W.H. Freeman and Co., New York (1980).

Immunoassays are also provided by the present invention. In general, ELISAs are the preferred immunoassays employed to assess a specimen. Both polyclonal and moloclonal antibodies can be used in the assays. The specific assay to be used can be determined by one skilled in the art.

The compounds of the present invention are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art. Additionally, when the compound is being administered orally, there must be included a compound for preventing the degradation of the kringle.

The compounds of the present invention can be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor can be treated conventionally with surgery, radiation or chemotherapy combined with the kringles of the present invention and then the kringles of the present invention can be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor. Additionally, the kringles of the present invention, fragments thereof, antisera, receptor agonists, receptor

antagonists, or combinations thereof, are combined with pharmaceutically acceptable excipients, and optionally sustained-release matrix, such as biodegradable polymers, to form therapeutic compositions. A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid/base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. The sustained-release matrix desirably is chosen from biocompatible materials such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (co-polymers of lactic acid and glycolic acid) polyanhydrides, poly(ortho)esters, polyproteins, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides, polyvinyl propylene, polyvinylpyrrolidone and silicone. A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid). The angiogenesis-modulating therapeutic composition of the present invention can be a solid, liquid or aerosol and can be administered by any known route of administration. Examples of solid therapeutic compositions include pills, creams, and implantable dosage units. The pills can be administered orally, the therapeutic creams can be administered topically. The implantable dosage unitst can be administered locally, for example at a tumor site, or which can be implanted for systemic release of the therapeutic angiogenesis-modulating composition, for example subcutaneously. Examples of liquid composition include formulations adapted for injection subcutaneously, intravenously, intraarterially, and formulations for topical and intraocular administration. Examples of aersol formulation include inhaler formulation for administration to the lungs. The the kringles of the present invention of the present invention also can be used to generate antibodies that are specific for the inhibitor and its receptor. The antibodies can be either polyclonal antibodies or monoclonal antibodies. These antibodies that specifically bind to

the the kringles of the present invention its receptors can be used in diagnostic methods and kits that are well known to those of ordinary skill in the art to detect or quantify the the kringles of the present invention or it's receptors in a body fluid or tissue. Results from these tests can be used to diagnose or predict the occurrence or recurrence of a cancer and other angiogenic mediated diseases. The the kringles of the present invention also can be used in a diagnostic method and kit to detect and quantify antibodies capable of binding the kringles of the present invention. These kits would permit detection of circulating antibodies which indicates the spread of micrometastases in the presence of the kringles of the present invention secreted by primary tumors in situ. Patients that have such circulating anti-antibodies can be more likely to develop multiple tumors and cancers, and can be more likely to have recurrences of cancer after treatments or periods of remission. The Fab fragments of these antibodies can be used as antigens to generate Fab-fragment antisera which can be used to neutralize anti the kringles of the present invention antibodies.

By way of background, tissue plasminogen activator (tPA) contains two kringle structures. Angiostatin, an angiogenic inhibitor, is composed of four kringles of plasminogen. Computer-aided analysis revealed similarities and differences between the tPA and plasminogen kringles. The tPA kringle 2 is composed of 86 amino acids (Asn^{177}-Thr^{263} in tPA molecule) and has been expressed in *E. coli* cells to 39.9% of the total cellular protein. The refolded peptide fragment specifically inhibited endothelial cell proliferation. Furthermore, a series of mutations in the tPA kringle 2 domains were created by PCR-mediated site-directed mutagenesis. Among seven mutations that have been examined, a substitution of histidine with tyrosine dramatically increased its anti-angiogenic activity. The improved properties associated with this modified tPA kringle 2, such as its lower molecular weight, higher anti-angiogenic activity and the ability to produce large quantities of proteins targets these proteins for treatment of diseases related to abnormal growth of

blood vessels.

The pharmaceutical composition of the present invention utilizes kringle proteins. There are at least three functional sites in a single short peptide (approximate 90 amino acids) of tPA kringle 2: a lysine-binding site, an endothelial cell-binding site and a possible site for anti-angiogenesis activity. To test the functions of tPA kringle 2 the kringle domains of tPA were cloned and expressed. The tPA kringle domains have been difficult to express because very small amounts of tPA kringle 2 (0.05 mg/g cells) are produced from *E. coli* and 3 ;I.9- only 0.3-0.5 mg/g cells of kringle 2 secreted from *Pichia* cells have been reported. However, the tPA kringle 2 peptide was successfully expressed as high as 58.5 mg/g cells *E. coli* according to the methods of the present invention. The recombinant tPA kringle 2 peptide inhibits the proliferation of endothelial cells. In addition. a potent active site in the tPA kringle 2 structure was discovered for expressing kringle anti-angiogenic activity.

As a thrombolytic agent, tPA has been used for treatment of stroke and myocardial infarction. It was further uncovered as part of the present invention that an internal fragment of tPA, kringle 2 possesses anti-angiogenic activity. The tPA kringle 2 shares significant homology with angiostatin, an endogenous angiogenic inhibitor. Angiostatin is an internal fragment of plasminogen. However, plasminogen is not a therapeutic protein. Accordingly, in order to obtain strong anti-angiogenic tPA molecule, PCR-mediated site-directed mutagenesis has been performed.

The benefits of using the kringles set forth in the present application include the following: (1) Large quantities of tPA kringle 2 can be mass produced in its modified versions in *E. coli* cells. Also, tPA kringle 2 can be over-expressed as high as 39.9% of total cellular proteins, more than thousand times higher than previously reported; (2) This is the first to

discovery of an internal fragment (Asn^{177}-Thr^{263}, kringle 2) of tPA which possesses anti-angiogenic activity; and (3) The substitution of histidine with an aromatic residue tyrosine significantly increases anti-angiogenic activity of tPA kringle 2 domain.

The present invention also provides a method of treating a disease. The methods includes the steps of administering an effective amount of a kringle protein, KED or tPA. Applications for the present invention include but are not limited to the following diseases: atherosclerosis, arthritis, retinopathy, cancer, and other similar diseases.

The method can be used in multiple applications. In using the present method a kit is provided containing therein all of the necessary components. In order to make this procedure as easy as possible for the individual, the kits include all of the necessary reagents for creating the anti-angiogentic activity in cells and in mammal. The kits can also include the specific kringles to be used by the individual. Alternative kits are made available in which the individual can create their own pharmaceutical composition based on the peptides provided by the present invention.

The kit provides instructions, antiserum, the proteins of the present invention, and possibly radiolabeled kringles and/or reagents for precipitation of bound antibody complexes. The kit is useful for the measurement of these kringles in biological fluids and tissue extracts of animals and humans with and without tumors. Another kit is used for localization of the kringles in tissues and cells. This kringle immunohistochemistry kit provides instructions, kringle antiserum, and possibly blocking serum and secondary antiserum linked to a fluorescent molecule such as fluorescein isothiocyanate, or to some other reagent used to visualize the primary antiserum. Immunohistochemistry techniques are well known to those skilled in the art. This kringle immunohistochemistry kit permits localization of kringle in tissue

sections and cultured cells using both light and electron microscopy. It is used for both research and clinical purposes. For example, tumors are biopsied or collected and tissue sections cut with a microtome to examine sites of kringle production. Such information is useful for diagnostic and possibly therapeutic purposes in the detection and treatment of cancer. Another method to visualize sites of kringle biosynthesis involves radiolabeling nucleic acids for use in in situ hybridization to probe for kringle messenger RNA. Similarly, the kringle receptor can be localized, visualized and quantitated with immunohistochemistry techniques.

Also provided by the present invention, are the peptides which are positives derived from the sequences of the present invention are as follows: peptide 10010281 (region 4 k2 R133-W142 k2tPA) Acetyl-RNPDGDAKPW-amide MW 1197 (10-mer) peptide 10010286 (region H2/5 H144-L151k2tPA) Acetyl-YVLKPRR-amide MW 973 (7-mer); peptide 10010284 (region K2 H144-L151k2tPA)) Acetyl-HVLKNRR-amide MW 964 (7-mer); hK2tPA (88aa) sequence begins at N177→T263 of tPA 1
It is important to note that the H mutant has a single point mutation H244→Y. The peptides are short 7-10 amino acid sequences which preserve the majority of the biologic activity of the parent H mutant protein, which is derived from the sequence of the second kringle domain (k2) of human tissue plasminogen activator (tPA) protein, specifically from amino acid 177 to 263.

The above discussion provides a factual basis for the use of kringles in the treatment of disease. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

EXAMPLES

GENERAL METHODS:

General methods in molecular biology: Standard molecular biology

techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988), and in Watson et al., *Recombinant DNA*, Scientific American Books, New York and in Birren et al (eds) *Genome Analysis: A Laboratory Manual Series, Vols. 1-4* Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). In-situ (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996, Blood 87:3822.)

General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

Immunoassays

In general, ELISAs are the preferred immunoassays employed to assess a specimen. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533;

3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989

Recombinant Protein Purification

Marshak et al, "Strategies for Protein Purification and Characterization. A laboratory course manual." CSHL Press, 1996.

For gene therapy:

By gene therapy as used herein refers to the transfer of genetic material (e.g DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense) whose production in vivo is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme, polypeptide or peptide of therapeutic value. Alternatively, the genetic material of interest encodes a suicide gene. For a review see, in general, the text "Gene Therapy" (Advances in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) *ex vivo* and (2) *in vivo* gene therapy. In *ex vivo* gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material *in situ*.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the

recipient organism *in situ*, that is within the recipient. In an alternative embodiment, if the host gene is defective, the gene is repaired *in situ* [Culver, 1998]. These genetically altered cells have been shown to express the transfected genetic material *in situ*.

The gene expression vehicle is capable of delivery/transfer of heterologous nucleic acid into a host cell. The expression vehicle can include elements to control targeting, expression and transcription of the nucleic acid in a cell selective manner as is known in the art. It should be noted that often the 5'UTR and/or 3'UTR of the gene can be replaced by the 5'UTR and/or 3'UTR of the expression vehicle. Therefore as used herein the expression vehicle may, as needed, not include the 5'UTR and/or 3'UTR of the actual gene to be transferred and only include the specific amino acid coding region.

The expression vehicle can include a promotor for controlling transcription of the heterologous material and can be either a constitutive or inducible promotor to allow selective transcription. Enhancers that can be required to obtain necessary transcription levels can optionally be included. Enhancers are generally any non-translated DNA sequence which works contiguously with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The expression vehicle can also include a selection gene as described herein below.

Vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*,

Butterworths, Boston MA (1988) and Gilboa et al (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods.

Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

A specific example of DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector AdenoTK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin as well as others. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir.

Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation will not occur.

Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions

of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The recombinant vector can be administered in several ways. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

An alternate mode of administration can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients or into the spinal fluid. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

Alternatively, transfection of promoter sequences, other than one normally found specifically associated with these kringles, or other sequences which would increase production of protein are also envisioned as methods of gene therapy. An example of this technology is found in Transkaryotic Therapies, Inc., of Cambridge, Mass., using homologous recombination to insert a "genetic switch" that turns on an erythropoietin gene in cells. See Genetic Engineering News, Apr. 15, 1994. Such "genetic switches" could be used to activate the kringles (or the receptor) in cells not normally expressing kringles (or the receptor) . Gene transfer methods for gene therapy fall into three broad categories-physical (e.g., electroporation, direct gene transfer and particle bombardment), chemical (lipid-based carriers, or other non-viral vectors) and biological (virus-derived vector and receptor uptake). For example, non-viral vectors can be used which include liposomes coated with DNA. Such liposome/DNA complexes can be directly injected intravenously

into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupffer cells. These cells are long lived and thus provide long term expression of the delivered DNA. Additionally, vectors or the "naked" DNA of the gene can be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Delivery of gene products/therapeutics (compound):

The compound of the present invention is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

In the method of the present invention, the compound of the present invention can be administered in various ways. It should be noted that it can be administered as the compound or as pharmaceutically acceptable salt and can be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. The compounds can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and direct parenchymal infusion techniques. Implants of the compounds are also useful. The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or

liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses can be single doses or multiple doses over a period of several days, but single doses are preferred.

The doses can be single doses or multiple doses over a period of several days. The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated.

When administering the compound of the present invention parenterally, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and

buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, microspheres and programmable pumps for parenchymal infusion into organs, such as brain. Examples of delivery systems useful in the present invention include: 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

A pharmacological formulation of the compound utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compounds in tablets, suspensions, solutions,

emulsions, capsules, powders, syrups and the like are usable. Known techniques which deliver it orally or intravenously and retain the biological activity are preferred.

In one embodiment, the compound of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used. The quantity to be administered will vary for the patient being treated and will vary from about 100 ng/kg of body weight to 100 mg/kg of body weight per day.

Additionally, these kringle proteins can be chemically coupled to isotopes, enzymes, carrier proteins, cytotoxic agents, fluorescent molecules, chemiluminescent, bioluminescent and other compounds for a variety of applications. The efficiency of the coupling reaction is determined using different techniques appropriate for the specific reaction. For example, radiolabeling of these protein with .sup.125 I is accomplished using chloramine T and Na.sup.125 I of high specific activity. The reaction is terminated with sodium metabisulfite and the mixture is desalted on disposable columns. The labeled protein is eluted from the column and fractions are collected. Aliquots are removed from each fraction and radioactivity measured in a gamma counter. In this manner, the unreacted Na.sup.125 I is separated from the labeled protein. The protein fractions with the highest specific radioactivity are stored for subsequent use such as analysis of the ability to bind to the antisera. Another application of protein conjugation is for production of polyclonal antisera. For example, the kringle proteins containing lysine residues are linked to purified bovine serum albumin using glutaraldehyde. The efficiency of the reaction is determined by measuring the incorporation of radiolabeled protein. Unreacted glutaraldehyde and protein are separated by dialysis. The conjugate is stored

for subsequent use. Antiserum against these kringles, analogs thereof, protein fragments and receptor can be generated. After protein synthesis and purification, both monoclonal and polyclonal antisera are raised using established techniques known to those skilled in the art. For example, polyclonal antisera can be raised in rabbits, sheep, goats or other animals. These kringle proteins conjugated to a carrier molecule, such as bovine serum albumin, is combined with an adjuvant mixture, emulsified and injected subcutaneously at multiple sites on the back, neck, flanks, and sometimes in the footpads. Booster injections are made at regular intervals, such as every 2 to 4 weeks. Blood samples are obtained by venipuncture, for example using the marginal ear veins after dilation, approximately 7 to 10 days after each injection. The blood samples are allowed to clot overnight at 4.degree. C. and are centrifuged at approximately 2400.times.g at 4.degree. C. for about 30 minutes. The serum is removed, aliquoted, and stored at 4.degree. C. for immediate use or at -20 to -90.degree. C. for subsequent analysis.

In addition to the diseases specifically stated herein, KED is also useful in the treatment of other diseases, such as but not limited to: bronchial vascular congestion, inflammatory bowel disease, adult respiratory distress syndrome, Castleman's disease, mycosis fungoides, atherosclerosis, restenosis, ischemia (limb, cerebral, myocardial), cancer, inflammation/chronic inflammatory disease, diabetes (diabetic retinopathy), ophthamology, wound healing, glomerulonephritis, Moyamoya disease, coronary artery disease, ulcer, arthritis, Kaposi sarcoma, psoriasis, peripheral arterial disease, avitreoretinopathy, Macular pucker, chronic patella tendinitis, fibrodysplasia ossifecaus progressiva, Chrohn's disease, hepatitis, neovascular glaucoma, aneurysm, rheumatoid arthritis, leiomyoma, Bartonella quintata infection, AIDS, aortic aneurysms, renal disease, veno-occulsive disease, allergic rhinitis, and hemangioma.

EXAMPLE 1

Overview of the Anti-angiogenic, KED Fusion Protein:

Human plasminogen kringle 5 is a novel angiogenesis inhibitor. It is a small protein of 10 kDa molecular weight. Angiostatin, a 38 kDa protein, contains 4 kringles of plasminogen kringle 1 through 4. A proliferation assay using bovine endothelial cells indicates that the plasminogen kringle 5 is at least as strong as anti-angiogenic effect as angiostatin. Endostatin is a newly identified angiogenic inhibitor and is the most effective inhibitor of proliferating endothelial cells so far discovered. Endostatin is a 20 kDa C-terminal fragment of collagen α1 (XVIII) sharing no homology with angiostatin and plasminogen kringle 5 domains. Hydrophobic profile analysis indicates that endostatin is highly hydrophobic and plasminogen kringle 5 is a hydrophilic protein. Refolding of endostatin protein produced from *E. coli* cells is not efficient (<1%). The cDNA of human plasminogen kringle 5 domain was fused with that of human collagen α1 (XVIII) C-terminal fragment and this generated a novel protein with improved (approximately 100 folds) refolding efficiency and significantly increased anti-angiogenic activity. The fusion protein, now called "KED" has an estimated MW of 30,001Da, has been abundantly produced in *E. coli* cells. Its anti-angiogenic activity has been examined in three ways: *in vitro* endothelial cell proliferation assay, *in vivo* corneal assay and *in vivo* tumor models.

Molecular Cloning & Protein Synthesis:

The KED gene was constructed by fusion of cDNA plasminogen kringle 5 encoding Glu478 -Ala562 with a collagen α1(XVIII) cDNA fragment encoding Hisl154 -Lys1336. The KED gene (Fig. I) is expressed in *E. Coli* cells. AGA codon is a rare codon used in *E. Coli* (Wada et al., 1990). The $tRNA_{AGA}$ is encoded by dnaY gene. The dnaY gene also has been reported to be essential for DNA replication (Garcia et al., 1986). To improve productivity of the KED protein in *E. Coli,* a vector, pEXdnaY, was constructed containing a strong T7 promoter for high- speed transcription of KED mRNA and a dnaY

gene supplying $tRNA_{AGA}$ for efficient translation of the KED product. The process for KED gene fusion and cloning in pEXdnaY vector is shown in the Sequence Listing.

The expression of KED protein from pEXdnaY -hKED, was induced by IPTG (isopropyl β-D-thiogalactopyranoside). Abundant KED production was achieved. KED protein accumulated up to 47% of total cellular protein by 2 hours of induction with 0.5 mM IPTG (Fig. 2). Optimal production of KED protein was performed using a fermentor. The KED protein was produced as inclusion bodies in cytosol. KED inclusion bodies were isolated and efficiently refolded in the presence of 0.5 M L-arginine at pH 8.6. As a control, human endostatin was simultaneously expressed from pEXdnaY-hED. More than 99% of the human endostatin protein precipitated in the refolding solution as has been previously observed. No precipitation of KED fusion occurs during the refolding process. This indicates efficient productivity of KED compared to that of endostatin.

Pure KED protein was obtained by HiTrap Heparin-Sepharose chromatography. Analysis of KED protein by denatured SDS-PAGE (polyacrylamide gel electrophoresis) revealed a single 30 kDa peptide that agreed with the molecular weight estimated from the deduced amino acid sequence (Fig. 3).

N-terminal analysis of KED protein (by Commonwealth Biotechnologies, Richmond, V A) provided an identical sequence of MEEDHMFGNGKGYRG as deduced from DNA sequence (Fig. 1), where the 5th residue is Cys that generally does not show up by the analysis system. Compositional analysis of KED generated similar results with those which were predicted. Both the N-terminal and compositional analysis indicated that the pure protein isolated is the correct form of the KED gene product.

Endothelial Proliferation:

A proliferation assay using bovine endothelial cells indicated that fusion protein KED completely inhibited the endothelial cells migration at a concentration as low as 8 nM (250 ng/ml), where, human endostatin shows almost no activity at 12 nM (250 ng/ml) by the same assay (Fig. 4). The IC_{50} of KED observed is less than 100 ng/ml. The IC_{50} of human endostatin in the assay was 350 ng/ml. These results indicate that the human fusion protein KED is much stronger than endostatin, the strongest anti-angiogenic agent reported to date.

Inhibition of Blood Vessel Growth in Rat Cornea:

Rodent corneal assay has been performed to test *in vivo* the efficacy of KED fusion protein. Different amounts, 1.25 ng, 5 ng, and 10 ng of KED were applied in the presence of 50 ng of bFGF 1.25 ng of KED fusion protein efficiently inhibited the growth of capillary blood vessels from the corneal bed (Fig. 5).

Suppression of Gliomas in Animal Model:

Gliomas were implanted in the rat brain, One mg/kg body weight of KED protein was used to treat animals for 14 days (Fig. 6).

Anti-angiogenic Mechanism of Kringle: Cloning and Expression of tPA Kringles:

The tPA kringles have been cloned and inserted into pET11a a vector for protein expression under the control of a strong T_7 phage promoter and the laclq repressor. Recombinant plasmid $pK1_{tPA}$ encodes r-[$K1_{tPA}$], where, r stands for recombinant and [$K1_{tPA}$] represents amino acid sequence residues [Ile86-Asn 177] of kringle 1 domain in tPA molecule. The recombinant plasmid $pK2_{tPA}$ encodes r-[$K2_{tPA}$] representing amino acid residues [Asn177-Thr263] of tPA kringle 2 domain. Recombinant plasmid $pK12_{tPA}$ encodes a r-[$K12_{tPA}$]

protein containing kringle 1 and kringle 2 domain, an internal fragment of [Ile86-Thr263] in tPA protein sequence.

Recombinant proteins expressed in *E. Coli* were analyzed by 16% polyacrylamide gel. Whole cellular proteins of 50 μg were denatured by SDS and β-mercaptoethanol and loaded into a gel for electrophoresis. Abundant expression of 10 kDa protein has been detected from the cells carrying $pK2_{tPA}$ construct (Fig. 7). 10 colonies of $pK2_{tPA}$, construct were selected and all ten expressed the same 10 kDa protein. A 20 kDa protein was found from the cells harboring $pK12_{tPA}$ plasmid. All ten clones of $p12_{tPA}$ expressed the 20 kDa protein. These two proteins were produced at very high levels that occupied as high as 39.9% of the total cellular protein by a density analysis of Coomassie blue-stained PAGE gel. However, no expression was observed from the cells of ten clones containing plasmid $pK1_{tPA}$. No expression of kringle 1 domain was confirmed by an additional screening of 20 pK 11_{tPA} clones.

The DNA deduced amino acids for r-[$K2_{tPA}$] protein is a peptide of 88 residues including the initial methionine. Molecular weight (MW) of r-[$K2_{tPA}$] estimated at of 9,716.42 Da, agrees with the size of 10 kDA analyzed from the polyacrylamide gel. r-[$KI2_{tPA}$] contains 179 residues with a MW of 19810.92 Da as expected from its DNA sequence. Expression of a 20 kDa protein from the $pK12_{tPA}$ plasmid perfectly fits with that expected MW. However, even r-[$K1_{tPA}$], sharing 92 identical amino acids with the N-terminus of r-[$K12_{tPA}$], was not expressed.

tPA kringle 2 was successfully expressed, as was the tPA kringle 1 plus kringle 2. However, tPA kringle 1 domain was unable to be expressed. Expression of tPA kringle 1 was only achieved by the fusion of kringle 2 domain. The peptide containing tPA kringle 1 only, has never been

expressed. Expression of tPA kringle 1 has been reported as a fusion of the plasminogen kringle domain (DeSerrano et al., 1992).

Endothelial proliferation assay:

Both recombinant proteins, r-[$K2_{tPA}$] and r-[$K12_{tPA}$], contain tPA kringle 2 domains. The tPA kringle 2 structure has the lysine-binding site. The lysine-bind site in the tPA kringle 2, binding resins coupled with lysine ligand, can be used for affinity protein purification. The stable epoxy-activated-lysine-agraose gel was used to purify the recombinant proteins r-[$_{K2tPA}$] and r-[$K12_{tPA}$]. L-arginine is a competitor of the lysine ligand. Efficient binding of recombinant tPA kringles with lysine ligand occurs, when the concentration of L-arginine in the protein solution is reduced below 25 mM. Elution of recombinant tPA kringles was carried out by using a competitive ligand of lysine, specifically 6-amino-n-caproic acid. There are three peaks detected in the eluted fractions from a 0-200 mM gradient. One is between 30 to 45 mM. One is 60 to 105 mM and a large peak between 125 to 150 mM of 6-amino-n-caproic acid. Samples from these fractions were subjected to SDS-PAGE analysis. The r-[$K2_{tPA}$] and r-[$K12_{tPA}$] proteins eluted in the peak of 125-150 mM 6-amino-n-caproic acid, appeared to be homogeneous on the denatured 16% polyacrylamide gel.

Different concentrations of recombinant tPA kringles were added into the culture of bovine capillary endothelial (BCE) cells. The cells were maintained in DMEM medium for five days. Cells which passed through an assay filter were counted. The assay results indicated that both the r-($K2_{tPA}$] and r-($K12_{tPA}$] proteins are active for anti-angiogenesis. Protein r-[$K2_{tPA}$] containing tPA kringle 2 alone showed stronger activity than r-[$K2_{tPA}$] suggesting that the tPA kringle 2 is an angiogenesis inhibitor (Fig. 8).

A Potent Anti-Angiogenic Active Site in Kringle:

Obtaining a kringle possessing strong anti-angiogenesis activity benefits the development of an anti-cancer agent. Identification of active site(s) for anti-angiogenesis activity of kringles leads to an understanding of the anti-angiogenesis mechanism. A way to address this is to perform *in vitro* site-directed mutagenesis in kringles. The tPA kringle domain is a good choice for the mutation for the following reasons: (1) the tPA kringle 2 contains a strong lysine-binding site that is preferred for ease of isolation of the kringle mutants; and (2) the tPA kringle 2 has been extensively studied.

All five plasminogen kringles have been cloned. The sequence of anti-angiogenic activity of plasminogen kringles is kringle 5>1>3>=2>4. The most active plasminogen kringles are kringle 5 and 1. Plasminogen kringle 4 did not show activity. Because performing the analysis of anti-angiogenic activity of tPA kringles did not include plasminogen kringles, the tPA kringle activity can not be compared with that of plasminogen kringles. Sites were chosen for mutation in tPA kringle using amino acid sequences of plasminogen kringles as references, in which, plasminogen kringle 5 and 1 were selected for the positive and kringle 2, 3, 4 especially kringle 4, were selected for the negative reference.

Ideally, positive selection or negative selection can be designed. First, the amino acid sequence of tPA kringle 2 is aligned with the five plasminogen kringles. Next, the position(s) are identified where the amino acid residue(s) is identical between plasminogen 5 and 1 but is different from plasminogen 4 and tPA kringle 2. If a position is occupied with a similar or the same amino acid residue in tPA kringle 2 and plasminogen kringle 4, where the residue should be mutated is the top priority. Then the amino acid residue(s) in kringle 2 is changed to an amino acid that is identical in plasminogen kringle 5 and 1. Finally, the resultant mutant is expressed and tested for its effects on endothelial proliferation to see if the mutation changes (increases) its anti-

angiogenic activity. Alternatively, negative selection can be designed by changing a residue in the tPA kringle identical with both of plasminogen kringle 5 and 1 to an amino acid identical with that of plasminogen kringle 4. Here in the present studies, mutations for positive selection were only performed.

The mutagenesis procedure utilizes a super-coiled, double-stranded and methylated DNA plasmid $pK2_{tPA}$ and two synthetic oligonucleotide primers containing the desired mutation. The PCR reaction employing Pfu, a proofreading DNA polymerase, efficiently produced a nicked, unmethylated daughter plasmid with a mutation which was created by the uptake of the oligonucleotide primers. The methylated parent $pK2_{tPA}$ plasmid was eliminated by restriction digestion using Dpn1, which is an enzyme, cutting the methylated substrate DNA. The mutated daughter plasmids were recovered by transformation into high-efficient competent *E. coli* cells. A control PCR accompanied each set of mutation thus excluding the oligodeoxynucleotide primers. Selection of transformants using the ampicillin resistance maker encoded by the vector indicated that at least a hundred fold of colonies were obtained from the PCR reaction mixture containing the mutation primers compared to that of PCR excluding the primers.

Dideoxynucleotide DNA sequencing of plasmids revealed that all designed mutations were successfully introduced on the tPA kringle DNA strand. Any undesired or unexpected mutation was not observed, showing that high fidelity DNA extension has been performed during the PCR process using the proof reading Pfu enzyme.

All kringle mutants are approximately 10 kDa MW. Expression of mutants were analyzed on 16% polyacryl amide gel electrophoresis (Fig. 9), four of them were expressed. However, the Y, N and G mutants were unstable. Interestingly, the Y, N and G mutants are all located in the middle

in the kringle structure, suggesting that a mutation on this region creates unstable mRNA or protein structure.

tPA kringle 2 mutants were solubilized and reduced with 8 M urea and 140 mM beta-mercaptoethanol. The mutants were then fractionated by Superfine Sephadex G- 75 chromatography in the resence of 8 M urea. The reduced mutant proteins appeared to be homogenous, as shown by the denatured SDS-PAGE analysis. The pure reduced mutants were refolded and pureed.

Kringle mutants S, K, H, W and the wild-type tPA kringle protein were tested by proliferation assay using bovine endothelial cells in the presence of bFGF. Mutations on K and W showed no effects on the anti-angiogenic activity of tPA kringle. The S mutation slightly effected the activity. The mutation on the H site generated at least 4 times higher anti-angiogenic activity compared to the parental wild-type kringle (Fig. 10), indicating that the H site is a potent active site for the expression of anti-angiogenic activity of the kringle structure.

EXAMPLE 2

Materials and methods

1. Adaptor anchored gene cloning

Human cDNA pool was generated by reverse transcription from liver and kidney mRNA using an oligodeoxynucleotide (dT)17 primer. The cDNAs were reverse transcribed in a 20 µl reaction solution containing 2.5 µg of human liver or kidney mRNA (Clontech, Palo Alto, CA), 200 ng of oligo (dT)17 primer, 5 mM DTT, 2.5 mM MgCl, 400 M of dNTP and 200 units of reverse transcriptase (BRL, Gaithersburg, MD) in 50 mM Tris buffer (pH 8.3). The mixture excluding reverse transcriptase was heated at 72° C for 5 minutesto denature mRNA and was quickly chilled on ice for 10 minutesallowing primer annealing. Then the contents were incubated at 42° C for 5 min, and

combined with 200 units of reverse transcriptase for an hour incubation. The cDNA fragments encoding plasminogen kringle 5 domain and collagen a1(XVIII) C-terminus were amplified by polymerase chain reaction (PCR).

At first, the cDNA fragment, without adaptors encoding plasminogen kringles 1 to 5, was amplified by a sense primer and a complementary primer: 5'-CTCTCAGAGTGCAAGACTGGGAATGGAAAGAAC (Leu100-Asn110), and 5'-GGCCGCACACTGAGGGACATCACAGTAGTC (Ala562-Asp553 according to the amino acid sequence of human plasminogen) (Folkman, 1996). A cDNA fragment encoding collagen a1(XVIII) C-terminus was amplified by a set of primers: 5'-CACAGCCACCGCGACTTCCAGCCGGTGCTC (His1154-Leu1163) for the sense 5'-end of the peptide, and 5'-CTACTTGGAGGCAGTCATGAAGCTGTTCTCAAT (Lys1336-Ile1327) for the complementary 3'-end (Folkman, 1996). Amplification of cDNA fragments performed by using a poof-reading thermostable Pfu DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction solution (100 µl) contains 20 mM Tris (pH 8.0), 2 mM MgCl, 10 mM KCl, 0.1% Triton X-100, 10 µl first strand cDNA from the above reverse transcriptional reaction, 300 ng primers and 5 units of Pfu DNA. Twenty-five reaction cycles consisting of 45 sec at 94° C for denaturing, 45 sec at 59° C for annealing and 3 minutesat 72° C for extension were performed. PCR products were analyzed by a 1.5% agarose gel.

Addition of adaptor to the amplified cDNA fragments was performed by PCR using adaptor anchored primers. Primers for cloning of the human plasminogen kringle 5 were 5'-GGAATTCCATATGGAAGAAGACTGTATGTTTGGG (G-[EcoRI]-[NdeI]-[Glu478-Gly486]), and 5'-GGAATTCCATATGGGCCGCACACTGAGGGACATC (G-[EcoRI]-[NdeI]-[Ala562-Asp556]). Collagen a1(XVIII) C-terminus with adaptors were amplified using primers 5'-

GGAATTCCATATGCACAGCCACCGCGACTTCCAG (G-[EcoRI]-[NdeI]-[His1154-Ile1160]), and 5'-CCGGGATCCCTACTTGGAGGCAGTCATGAAGCT (CCG-[BamHI]-[STOP]-[Lys1336-Ser1330). PCR reaction solution (100 µl) contains 50 mM Tris (pH 8.8), 2 mM MgCl, 10 mM KCl, 10 µl of the above resulted PCR reaction mixture containing the cDNA fragments, 250 ng of each primer and 7.5 units of Pfu DNA polymerase. Ten reaction PCR cycles consist of 45 sec at 94° C for denaturing, 45 sec at 37° C for annealing and 2 minutesat 68° C for DNA synthesis.

A 0.57 kb DNA fragment encoding 183 amino acid residues of collagen a1(XVIII) C-terminus with adaptors and a 0.28 kb fragment encoding 85 amino acid residues of plasminogen kringle 5, were purified from the PCR reaction mixture by spin column, and were inserted into expression vector pEXdnaY.

2. Vector pEXdnaY for expression of mammalian genes

A vector for the purpose of mammalian gene expression in *E. coli* was constructed. Vector pEXdnaY was constructed by combining of pET11a (Stratagene, La Jolla, CA) and pDC952. Five µg of pET11a plasmid (4.64 kb) was digested with 20 units of HindIII enzyme at 37° C for 1 hour. The linearized pET11a was treated by adding 32 units of calf intestinal alkaline phosphatase (CIAP) into the restriction reaction mixture and was incubated at 30° C for 30 min, to prevent self-ligation of pET11a. The linearized and dephosphorylated pET11a DNA was then ligated with a 632 bp HindIII-HindIII DNA fragment containing dnaY gene (tRNA for AGG and AGA) that was isolated from pDC952 plasmid by HindIII-digestion. The ligation solution was transformed into competent *E. coli* TG1 cells. Recombinant plasmid pEXdnaY were isolated and identified.

3. Gene fusion

A 558 bp cDNA fragment encoding collagen a1(XVIII) C-terminus flanked by NdeI and BamHI adaptors were cloned into the pEXdnaY vector, generating a plasmid pEXdnaY-hED. Then, a 255 bp cDNA fragment encoding plasminogen kringle 5 with NdeI adaptor at the two ends were fused in front of the collagen a1(XVIII) C-terminal fragment, generating plasmid pECdnaY-hKED. Briefly, 10 µg of pEXdnaY DNA was double-digested with 100 units of NdeI and 150 units of BamHI enzymes, following dephosphorylation with 64 units of CIAP. Pure dephosphorylated pEXdnaY vector was obtained from 0.7% agarose gel. The linearized vector was ligated with NdeI-BamHI DNA fragment of collagen a1(XVIII) C-terminus created by anchored PCR and was restriction digested with NdeI and BamHI enzymes. Recombinant plasmid pEXdnaY-hED was selected in TG1 cells and analyzed by double digestion of NdeI and BamHI enzymes. The resulted pEXdnaY-hED plasmid (5 µg) was further linearized by 100 units of NdeI and dephosphorylated with 32 units of CIAP. An NdeI-NdeI cDNA fragment encoding human plasminogen kringle 5 generated by NdeI-adaptor anchored PCR amplification was inserted into the NdeI site of pEXdnaY-hED by ligation with NdeI-linearized pEXdnaY-hED DNA. The ligation solution was transformed into competent *E. coli* TG1 cells. A recombinant plasmid pEXdnaY-hKED was identified by NdeI digestion and analysis of expression of a 30 kDa fusion protein.

4. Production of KED fusion

Plasmid pEXdnaY-hKED was transformed into *E. coli* BL21 (DE3) cells. A single ampicillin resistance colony was picked up and inoculated in 1 ml of LB media containing 100 g/ml of ampicillin. After 12 hours incubation at 37° C, the culture was diluted 100 fold into the same medium. Incubation with shaking at 250 rpm was continued until OD reached 0.45 at 600 nm. Optimal conditions for expression of KED fusion were investigated by induction with IPTG (isopropyl-D-thiogalactopyranoside) with different concentrations from

0.1 to 10 mM and varied time from 15 minutesto 3 hours. The induced cultures were quickly chilled on ice and centrifuged. Cells were washed with ice-cold PBS (phosphate saline buffer) containing 5 mM EDTA (ethylenediaminetetraacetic acid). An aliquot of cells, equivalent to 50 µg protein was used for analysis of KED fusion expression by denatured 10% polyacrylamide gel.

Optimal production of KED fusion was performed using a fermentor (Bioflo2000, New Brunswick Scientific, Edison, NJ). Over night culture (200 ml) was added into 8 l LB medium enriched with 25 g/l yeast extract. One ml of Antifoam 289 (Sigma, St. Louis, MO) was added to the medium to prevent foam-formation during fermentation. Agitation was kept 225 rpm through out the process. Airing was settled to 8 l/min until cells grown to OD_{600} 0.505. IPTG was added into the fermentor at the final concentration of 0.5 mM. Airing was changed to 20 l/min. After 1 hour 55 minutesproduction (from the time IPTG added), fermentation was terminated by adding 100 ml of 0.5 M EDTA (the final OD_{600} was 3.1). The culture was chilled on ice and collected filtration system using 0.04 µm polyethersulfone membrane (North Carolina SRT, Cary, NC). The cells (15.2 g of wet weight) were washed with 5 l of PBS containing 1 mM EDTA, and suspended in 500 ml of the same buffer containing 0.5% of NP-40 detergent. Cells were disrupted by passing a French Press Cells at 15,000 psi. Crude KED inclusion bodies were isolated by centrifugation for 20 minutesat 12,100 g at 4° C, and washed twice with ice-cold 500 ml PBS and 0.5% NP-40. Finally, the KED inclusion bodies (9.30 g wet weight, 1.60 g protein) were suspended in 50 ml of PBS.

5. Refolding of KED fusion

The NP-40 washed KED inclusion bodies was analyzed by denatured SDS-PAGE. Contents of KED in the inclusion bodies were quantitated by scan of Comassie blue stained gel (77.2% KED). To solubilize KED inclusion bodies, 50 g of solid urea and 1.2 g of Tris were added into 50 ml of KED

suspension. The pH was adjusted to 8.6, followed by adding of 80 µl Tween-20, 160 µl 0.5 M EDTA and 950 µl b-mercaptoethanol. The solution was incubated at 37° C for 1 hour 30 min, and centrifuged for 20 minutesat 17,400 g at 23° C. The resulted clear KED protein solution was carefully added (10 ml/min) in to 5:l of refolding solution containing 2.4 g of reduced glutathione (GSH). And that was immediately combined with another 5:l of refolding solution containing 0.5 g of oxidized glutathione (GSSG). The 10:l refolding solution used consisted of 1 kg L-arginine-HCl, 120 g Tris, 3.7 g EDTA, 10 ml of Tween-20 at pH 8.6. Refolding reaction was performed for 22h at 23° C.

Refolded KED solution was chilled on ice and concentrated to 650 ml by ultra-filtration using a regenerated cellulose membrane with 5 kDa weight cut-off (North Carolina SRT, Cary, NC). Precipitation of protein during ultra-filtration (16h) was excluded by centrifugation. The concentrated KED protein solution was further concentrated to 32 ml in a dialysis tube (3,500 Da molecular weight cut-off) and solid polyethylene glycol (PEG-8000). The pH of concentrated KED protein solution (32 ml at pH 8.5) was changed to pH 7.4. A quick passing of pI (pH 8.1) of KED was achieved by adding it drop by drop to 15 ml of 0.1 M sodium phosphate (pH 7.4) with simultaneous adding of 2N HCl to the mixture. Then, the KED protein solution was dialyzed against 5:l of 10 mM sodium phosphate (pH 7.4) containing 15 mM NaCl. Buffer was changed three times in 24 hours at 4 C. Dialyzed KED solution (145 ml) was centrifuged, and used for chromatography.

6. Heparin-Sepharose affinity chromatography

Twenty ml of dialyzed KED protein solution was diluted in 180 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl. The protein solution was loaded on a Heparin-Sepharose column (HiTrap pre-packaged 5 ml column, Pharmacia, Piscataway, NJ). Columns were washed with the same buffer until the OD_{280} returned to the base line. KED protein was eluted by a NaCl gradient (150-1000 mM). Pure KED protein eluted within 210-330

mM NaCl was identified by 10% polyacrylamide gel electrophoresis.

7. Acticlean-Etox chromatography

Acticlean Etox (Sterogene, Arcadia, CA) resins were cleaned with 1 M NaOH for 12 hours at 4° C, and packed to column. The column was washed with PBS until pH below 7.6. Pure KED protein was dialyzed against PBS, and loaded on Actilean Etox column. Each ml of Acticleas Etox gel was used for treatment 5 mg of KED protein. The KED protein was then sterilized by passing a 0.2 µm filter.

8. Endothelial cell proliferation assay

Bovine capillary endothelial cells (BCE cells, Folkman, 1996) were obtained for the proliferation assay (O'Reilly et al., 1997). Cells were washed with PBS and dispersed in a 0.05% trypsin solution. A cell suspension (25,000 cells/ml) was made with DMEM + 10% BCS and plated on 24-well culture plates (0.5 ml/well) and incubated at 37° C in 10% CO_2 for 24h. The medium was replaced with 0.5 ml DMEM + 5% BCS + 3 ng/ml recombinant bFGF and the test sample applied. After 72 hours , cells were dispersed in trypsin, resuspended and counted using a Coulter counter. Results were plotted as a dose-response and compared with other agents.

9. Endothelial cell migration assay

To determine the ability of the test compounds to block migration of endothelial cells toward the angiogenic factor bFGF, migration assays were performed in a modified Boyden chamber using BCE cells (Folkman, 1996). Cells were grown in DMEM supplemented with 10% FBS and supplemented at passage 15. To assess migration, the cells were starved overnight in DMEM supplemented with 0.1% BSA, harvested, suspended in DMEM/BSA, plated at 10^6 cells/ml on the lower surface of a gelatinized membrane (Nucleopore Corp., Plesanton, CA) in an inverted Boyden chamber and incubated for 1.5 hours to allow cell attachment. The chambers were then

righted, test material was added to the top well and incubated for 5 hours . Membranes were then fixed and stained and the number of cells migrating to the top of the filter in 10 high-powered fields were counted. DMEM with 0.1% BSA was used as a negative control and bFGF at 10 ng/ml was used as a positive control.

10. Rat corneal angiogenesis

The rat corneal assay was performed as described (Folkman, 1996). Five µl Hydron pellets (Hydron Laboratories, New Brunswick, NJ) containing 10 or 20 µg/ml bFGF (25 or 50 ng) plus 5 or 25 ng test compound and 10 µg/ml sulcrafate were implanted in a corneal pocket of anesthetized rats. After 7 days, the animals were sacrificed, perfused with colloidal carbon, and the excised corneas were mounted for microscopy, photography and analysis.

Results

1. Molecular cloning & protein synthesis

The KED gene was constructed by fusion of cDNA plasminogen kringle 5 encoding Glu478-Ala562 with a collagen a1(XVIII) cDNA fragment encoding His1154-Lys1336. The KED gene (Fig 1) is expressed in *E. coli* cells. AGA codon is a rare codon used in *E.coli* (Folkman, 1996). The $tRNA_{AGA}$ is encoded by dnaY gene. The dnaY gene also has been reported to be essential for DNA replication (Folkman, 1996). To improve productivity of KED protein in *E. coli*, a vector pEXdnaY was constructed containing a strong T7 promoter for high-speed transcription of KED mRNA and a dnaY gene supplying $tRNA_{AGA}$ for efficient translation of KED product. The process for KED gene fusion and cloning in pEXdnaY vector is shown in the Sequence Listing.

Expression of KED protein from pEXdnaY-hKED, was induced by IPTG (isopropyl -D-thiogalactopyranoside). Abundant KED production was achieved. KED protein accumulated to 47% of total cellular protein by 2 hours

induction with 0.5 mM IPTG (Fig 2). Optimal production of KED protein was performed using a fermentor. The KED protein was produced as inclusion bodies in cytosol. KED inclusion bodies were isolated and efficiently refolded in the presence of 0.5 M L-arginine at pH 8.6. As a control, human endostatin was simultaneously expressed from pEXdnaY-hED. More than 99% of human endostatin protein precipitated in the refolding solution has been previously observed (O'Reilly et al., 1997). No precipitation of KED fusion occurs during the refolding process, indicating efficient productivity of KED compared to endostatin.

Pure KED protein was obtained by HiTrap Heparin-Sepharose chromatography. Analysis of KED protein by denatured SDS-PAGE (polyacrylamide gel electrophoresis) revealed a single peptide of 30 kDa that agreed well with the molecular weight estimated from the deduced amino acid sequence (Fig 3).

N-terminal analysis of KED protein (by Commonwealth Biotechnologies, Richmond, VA) resulted in the same sequence MEEDHMFGNGKGYRG as deduced from the DNA sequence (Fig 1), where the 5th residue is Cys. Compositional analysis of KED generated similar results with that predicted. Both of N-terminal and compositional analysis indicates that the pure protein isolated is the correct form of the KED gene product.

2. Endothelial cell proliferation

Proliferation assay using bovine endothelial cells indicated that the fusion protein KED completely inhibited endothelial cell proliferation in a concentration-dependent fashion, with inhibitory activity as low as 8 nM (250 ng/ml) Human endostatin shows almost no activity at 40 nM (600 ng/ml) by the same assay (Fig 4a). These results indicate that the human fusion protein KED is a more potent inhibitor of endothelial cell proliferation than endostatin

(O'Reilly et al., 1997).

3. Endothelial cell migration

Migration assay using bovine endothelial cells indicated that the fusion protein KED completely inhibited endothelial cell migration at a concentration as low as 8 nM (250 ng/ml), where, human endostatin shows almost no activity at 12 nM (250 ng/ml) by the same assay (Fig 4b). The ic_{50} of KED is less than 100 ng/ml. IC_{50} of human endostatin in the assay was 350 ng/ml. These results indicate that the human fusion protein KED is a several-fold more potent inhibitor of endothelial cell migration than endostatin (O'Reilly et al., 1997).

4. Inhibition of rat corneal blood vessel growth

Rodent corneal assay was performed to test the *in vivo* efficacy of KED fusion protein (Fig 5). 12.5 ng of KED were applied in the presence of 25 ng of bFGF. 12.5 ng of KED fusion protein (Fig. 5b) efficiently inhibited the growth of capillary blood vessel from corneal bed compared with control, containing bFGF alone (Fig. 5a).

Discussion

Although anti-angiogenic activity varies in different kringle domain peptides, the triple disulphide bond kringle structure represents a new family of angioginhibitory compounds. Endostatin, a highly basic protein fragment of collagen a1(XVIII), possesses high affinity with heparin. Other anti-angiogenic proteins such as platelet factor-4 (Folkman, 1996) and thrombospondin-1 (Folkman, 1996) also show high affinity with heparin. It is not yet known that how heparin affinity contributes to the anti-angiogenic function of these proteins. However, heparin affinity is a common characteristic among these proteins. Proteins exhibiting heparin affinity seem to be a different class of angiogenic inhibitors compared to the kringle family. Plasminogen kringle 5 is the most anti-angiogenic of the kringle structures. Endostatin has the highest

anti-angiogenic activity within the heparin affinity proteins. In this study, these two anti-angiogenic members were fused into a single molecule. A cDNA fragment of 0.82 kb consisting of human plasminogen kringle 5 for the 5-prime end followed by the endostatin coding sequence was constructed. The cDNA has been successfully over-expressed in *E. coli*. The expressed product has been confirmed to be the designed fusion form, based on N-terminal sequencing and amino acid compositional analysis. A series of anti-angiogenic studies such as, endothelial proliferation and corneal assay have been performed to test the properties of this fusion protein. As expected, the fusion protein showed significantly higher anti-angiogenic activity than either kringle 5 or endostatin. Higher activity of this chimera reflects expression of combined activity from kringle 5 and endostatin. There is no structural homology between kringle 5 and endostatin. Thus, they exhibit anti-angiogenic activity by different mechanisms. Higher anti-angiogenic activity of this fusion protein is a result of simultaneous activation of different anti-angiogenic mechanisms. These results support the hypothesis that a fusion molecule of angiogenic inhibitors has, at least, additive benefit. The current study also demonstrated efficient protein production. Very inefficient production of endostatin in *E. coli* has been reported (O'Reilly et al., 1997). More than 99% of endostatin precipitates during refolding process. In the contrast, the fusion protein is completely soluble in the refolding solution. This improved productivity is another and unexpected advantage of gene fusion derived from the current study.

EXAMPLE 3

The kringles of the present invention can be: (i) Administered to tumor-bearing humans or animals as anti-angiogenic therapy; (ii) Monitored in human or animal serum, urine, or tissues as prognostic markers; and (iii) Used as the basis to analyze serum and urine of cancer patients for similar angiostatic molecules. It is contemplated as part of the present invention that the kringles can be isolated from a body fluid such as blood or urine of

patients or the kringles can be produced by recombinant DNA methods or synthetic protein chemical methods that are well known to those of ordinary skill in the art. Protein purification methods are well known in the art and a specific example of a method for purifying the kringles, and assaying for inhibitor activity is provided in the examples below. Isolation of human endogenous kringles is accomplished using similar techniques.

One example of a method of producing kringles using recombinant DNA techniques entails the steps of identifying and purifying kringles as discussed above, determining the N-terminal amino acid sequence of the purified inhibitor, synthetically generating 5' and 3' DNA oligonucleotide primers for the kringle sequence, amplifying the kringle gene sequence using polymerase, inserting the amplified sequence into an appropriate vector such as an expression vector, inserting the gene containing vector into a microorganism or other expression system capable of expressing the inhibitor gene, and isolating the recombinantly produced inhibitor. Appropriate vectors include viral, bacterial and eukaryotic (such as yeast) expression vectors. The above techniques are more fully described in laboratory manuals such as "Molecular Cloning: A Laboratory Manual" Second Edition by Sambrook et al., Cold Spring Harbor Press, 1989. The DNA sequence of human plasminogen has been published (Browne, M. J., et al., "Expression of recombinant human plasminogen and aglycoplasminogen in HeLa cells" Fibrinolysis Vol.5 (4). 257-260, 1991) and is incorporated herein by reference. The gene for these kringles can also be isolated from cells or tissue (such as tumor cells) that express high levels of the kringles by isolating messenger RNA from the tissue, using reverse transcriptase to generate the corresponding DNA sequence and then using the polymerase chain reaction (PCR) with the appropriate primers to amplify the DNA sequence coding for the active kringle amino acid sequence. Yet another method of producing kringles, or biologically active fragments thereof, is by protein synthesis. Once a biologically active fragment of a kringles is found using the assay system

described more fully below, it can be sequenced, for example by automated protein sequencing methods.

Alternatively, once the gene or DNA sequence which codes for kringles is isolated, for example by the methods described above, the DNA sequence can be determined using manual or automated sequencing methods well know in the art. The nucleic acid sequence in turn provides information regarding the amino acid sequence. Thus, if the biologically active fragment is generated by specific methods, such as tryptic digests, or if the fragment is N-terminal sequenced, the remaining amino acid sequence can be determined from the corresponding DNA sequence. Once the amino acid sequence of the protein is known, the fragment can be synthesized by techniques well known in the art, as exemplified by "Solid Phase Protein Synthesis: A Practical Approach" E. Atherton and R. C. Sheppard, IRL Press, Oxford, England. Similarly, multiple fragments can be synthesized which are subsequently linked together to form larger fragments. These synthetic protein fragments can also be made with amino acid substitutions at specific locations to test for agonistic and antagonistic activity in vitro and in vivo.

Protein fragments that possess high affinity binding to tissues can be used to isolate the receptor on affinity columns. Isolation and purification of the receptor is a fundamental step towards elucidating the mechanism of action of the kringles. Isolation of a kringle receptor and identification of the agonists and antagonists will facilitate development of drugs to modulate the activity of the receptor, the final pathway to biological activity. Isolation of the receptor enables the construction of nucleotide probes to monitor the location and synthesis of the receptor, using in situ and solution hybridization technology. Further, the gene for the receptor can be isolated, incorporated into an expression vector and transfected into cells, such as patient tumor cells to increase the ability of a cell type, tissue or tumor to bind and inhibit local angiogenesis.

The kringles of the present invention are effective in treating diseases or processes that are mediated by, or involve, angiogenesis. The present invention includes the method of treating an angiogenesis mediated disease with an effective amount of these kringles, or a biologically active fragment thereof, or combinations of angiostatin fragments that collectively possess anti-angiogenic activity, or agonists and antagonists. The angiogenesis mediated diseases include, but are not limited to, solid tumors; blood born tumors such as leukemias; tumor metastasis; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. The kringles of the present invention are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, Crohn's disease, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. These kringles can be used as a birth control agent by preventing vascularization required for embryo implantation. Kringles are useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (Rochele minalia quintosa) and ulcers (Helicobacter pylori). The synthetic protein fragments of these kringles have a variety of uses. The protein that binds to the receptor with high specificity and avidity is radiolabeled and employed for visualization and quantitation of binding sites using autoradiographic and membrane binding techniques. This application provides important diagnostic and research tools. Knowledge of the binding properties of the receptor facilitates investigation of the transduction mechanisms linked to the receptor. In addition, labeling these proteins with short lived isotopes enables visualization of receptor binding

sites in vivo using positron emission tomography or other modern radiographic techniques to locate tumors with the binding sites.

EXAMPLE 4

Construction of HED Fusion Protein:

The tPA kringle 2 carrying mutation of His244 → Tyr, is fused to collagen α1 (XVIII) C-terminus. A 558 bp DNA fragment encoding 183 amino acid residues of human collagen α1 (XVIII) C-terminus with adaptors that are amplified using primers 5'-GGAATTCCATATGCACAGCCACCGCGACTTCCAG (G-[EcoRI]-[NdeI]-[His 1154 → Gln 1160]), and 5'-CCGGGATCCCTACTTGGAGGCAGTCATGAAGCT (CCG-[BamHI]-[STOP]-[Lys1336 → Ser1330). A 287 bp cDNA fragment encoding 87 residues of tPA kringle 2 mutant H are amplified by primers 5'-GGAATTCCATAACAGTGACTGCTACTTTGGG (G-[EcoRI]-[NdeI]-[Asn177 → Gly183]), and 5'-GGAATTCCATATGGGTGGAGCAGGAGGGCACATC (G-[EcoRI]-[NdeI]-[Thr263 → Asp257]).

The collagen α1 (XVIII) C-terminus flanked by NdeI and BamHI adaptors are cloned into pEXdnaY vector. Then, cDNA fragment encoding tPA kringle 2 mutant H with NdeI adaptor in both ends are fused in the front NdeI site of collagen α1 (XVIII) C-terminus fragment, generating plasmid pEXdnaY-hHED. The estimated fusion HED 1 protein MW is 30.087 kDa.

EXAMPLE 5

To Inhibit Glioma Cell Invasion in the Rat Brain Using KED:

Neuropharmacology and efficacy of KED in xenograft glioma model.

Using validated culture and implantation techniques, nude rats (Cr:NIH-rnu) harboring intracranial human glioma xenografis (U251 Mgn) are treated with daily oral doses of KED for a period of 28 days beginning 1 week following implantation. Beginning 2 weeks following implantation, 8 animals

in each of the control and treatment groups and are sacrificed for histochemical and *in situ* detection protocols for quantitation. Subsequent groups of 8 animals in control and treatment groups are sacrificed weekly for a total of 4 sessions, a total of 6 weeks following implantation. An active drug concentration is determined in initial studies and dosage is adjusted if necessary, before proceeding. Further treatments are continued at the same dose only if adequate target KED concentrations have been achieved; otherwise a dose escalation of 25% is undertaken and the process repeated. A randomized cohort of animals implanted are then treated and survival studies undertaken. Results are fully duplicated in a repeat set of animals with a second xenografted human glioma cell line (U87MG).

Cell lines:

The U251MG cell line is originated by Ponten from a patient with a glioblastoma. The culture is initially mycoplasma positive and is cured by antibiotic treatment in culture (negative since 1987). U87MG is a cell line that is initiated from an anaplastic astrocytoma. All cell lines are routinely mycoplasma tested and have tested free of contamination. Cells are routinely maintained in DMEM +10% fcs in a 37°C humidiated incubator in 10% CO_2.

Intracranial nude rat xenograft:

Following CEAC guidelines in an approved animal use protocol, nude rats are inoculated intracerebrally as follows:

Cell culture:

Cells are harvested from 90% confluent culture plates by treatment with a 0.1% trypsin/EDTA for 10 minutes. Cells are rinsed off the plates with media and spun for 10 minutes at 1000 rpm. The cell pellet is resuspended in 100 μL of culture media, counted and the concentration of cells ad.iusted to 5 x $10^5$5μL. Viability of the cells is tested using Trypan blue exclusion counting on a Hausser Scientitic Brightline hemocytometer.

Implantation:

Animals are anesthetized with 100 mg/kg ketamine, 1.5 mg/kg xylazine, and 0.05 ml atropine i.m. The surgical zone is swabbed with betadine solution, the eyes coated with lacritube and immobilized in a small animal stereotactic device (Kopf, Cayunga, *CA)* After draping, a 1 cm incision is made 2 mm to the right of the midline 1 mm retro-orbitally, the skull exposed with cotton-tip applicators and a HP-4 dental drill bit is used with a micromanipulator to drill a hole 3 mm to the right of the bregma, taking care not to penetrate the dura. A #270 0 μL Hamilton syringe with a #4 point, 26s gauge needle containing tumor cell suspension [500.000 viable cells] is lowered to a depth of 3.5 mm, then raised to a depth of 2.5 mm. The cell suspension is injected stepwise at a rate of 0.5 μL/10 seconds until the entire volume had been injected. During and after the injection, careful note is made of any reflux from the injection site. After completing the injection, there is a waiting period for 2-3 minutes before withdrawing in a stepwise manner. The surgical zone is flushed at least twice with sterile saline and the hole sealed with bone wax. Finally, the skull is swabbed with Betadine before suturing the skin over the injection site. The animals are maintained for 1 week prior to treatment.

Drug administration schedule:

Using a 22 g stainless steel feeding tube, 18.75 mg/100 μl Saline (150 mg/kg 125 gm/animal) is administered orally to treated rats daily for 28 days, beginning 1 week following tumor establishment. This dose has generated serum concentrations of 10-15 μM in other studies and a target concentration of 5-15 μM in tissue is desired. Control animals receive identical handling except for oral administration of vehicle (SALINE) alone. In the survival cohorts, treatment is given daily until death of the animal, again beginning 1 week following implantation.

Tissue Collection and Processing:

At sacrifice, animals are anesthetized with ketamine/xylazine. CSF is removed via suboccipital puncture for HPLC [KED] using a 20 g needle; and after a blood specimen is taken, cardiac perfusion is performed with 250 mL sterile saline followed by fixation with 250 mL of 10% buffered formalin phosphate. Finally, the brain is removed and immersed in formalin for 24 hours. It is then placed in a 200-400 g coronal rat-brain matrix (Activational Systems Inc., Warren, MI) and cut into 1 mm blocks (same slice thickness as MRI) and subsequently imaged for histologic registration to MRI. Blocks grossly containing and adjacent to tumor are processed and paraffin embedded. In order to achieve accurate registration with MRI slices, the plane of section is made to correspond to that of the MRI, using anatomical landmarks as closely as possible. The embedded blocks are cut into serial 8μm sections onto uncoated slides for routine hematoxylin and eosin (H&E) staining and onto poly-L-lysine coated slides for immunohistochemical and ISH.

Histologic Staining:

Conventional H&E staining is undertaken on slides from tumor and adjacent blocks. ISH and immunohistochemistry are performed on immediately adjacent sections.

Alu *in situ* hybridization:

In situ hybridization is carried out as described (Appendix, SD). For this 8 μm adjacent serial sections are cut and mounted on pretreated glass slides. Pretreatment of glass slides included acid-cleaning, treatment with Denhardt's medium, acetylation and dipping in dilute Elmer's glue solution (1:100). Slides are deparaffinized in 3 changes of 100% xylene and rehydrated in graded concentrations of water in ethanol, air dried and stored at room temperature. The human Alu probe Blur 2, from Dr. Carl Schmid of the University of California at Davis, is constructed in a pUC plasmid inserted in

the Bam H1 site and represents a consensus sequence for human Alu repeat elements[115]. Random primed probes are labeled with UTP-digoxtgenin (dig) and visualized with an anti-digoxigenin polyclonal antibody conjugated to alkaline phosphatase are prepared as outlined in the Boehringer-Mannheim Genius™ Nonradioactive DNA Labeling and Detection Kit (Cat. No. 1093 657). Briefly, the slides are placed in 200 mM HCl for 20 minutes followed by a brief dip in H_2O, transferred to 2xSSC at 70°C for 30 minutes, followed by a brief dip in H_2O and incubated for 15 minutes at 37°C in 1 mg/ml proteinase K in 20 mM Tris with 2 mM $CaCl_2$, pH 7.4, followed by two 5-minute washes in H_2O. To link DNA to the nucleus, they are incubated in neutral 5% paraformaldehyde for 1 hour, then washed twice in 2xSSC for 5 minutes to block non-specific binding sites. The slides are then prehybridized at room temperature in the following cocktail: 5xSSC. 5% blocking regent (Boehringer-Manheim), 0.5% sarkosyl, 0.02% sodium dodecyl sulfate, 0.2 mg/ml sheared salmon sperm DNA and 50% formamide. The prehybridization solution is placed over the specimens and the slide placed in a humidity box for 30 minutes, the slides are rinsed twice in 2xSSC. DNA is denatured in 70% formamide-2xSSC for 5 minutes at 70°C followed by transfer to ice-cold H_2O to keep it single-stranded. The section is dehydrated through 70% and 100% ethanol. After air drying, the sample is hybridized overnight at room temperature with 0.4 ng/μl dig-labeled 300 bp Alu probe in the cocktail used for prehybridization. The sample is then washed twice in 2xSSC for 5 minutes each, followed by two washes at 55°C in 2xSSC for 15 minutes each. Immunological detection of the dig-labeled probe is done with dig-specific antiserum is performed as described in the Genius™ Kit. Stained slides are digitized for quantitation.

Statistical Considerations: Inhibition of human glioma invasion in rat brain using KED.

This is a two-sample, placebo-controlled trial in tumor-bearing rats of (KED) where the placebo is saline, the vehicle for administration of KED. The

primary endpoint is tumor volume, the margins measured as the infiltration border. There is a complete replication of the trial for each cell line. U251 MGn and U87MG. For each replication, 64 nude rats are inoculated intracerebrally with tumor cells. After one week of maintenance, half the rats are randomly treated with KED and half will receive saline. Eight randomly selected animals from each treatment group is sacrificed at 1 week intervals following treatment and the volume of tumor is measured.

Treatment of colon cancer by KED: Cell lines

Parental colon cancer cell line LS 174T was derived from a well-differentiated Human colonic adenocarcinoma (Moose et al., 1996), and has been characterized extensively, including its ability to product mucin *in vitro* (Standker et al., 1997), and when grown subcutaneously in athymic nude mice (Patthy, 1985). Cells injected into the cecal wall of nude mice produce well-differentiated primary colon cancers with little metastic ability (O'Reilly et al., 1993).

LS LiM 6, a derivative of LS174T with high liver-metastasizing ability during cecal growth, was established by serially selecting cells which metastasized from cecum to liver as previously described (O'Reilly et al., 1993). These cells form well-differentiated mucinous carcinomas in nude mice, with large glands containing PAS-positive (Folkman, 1996) mucin comprising ~50% of the tumor area. HM7 and LM12 are variants of LS174T which produce high and low amounts of mucin, respectively. Relative to the parental cell line, and were selected by a replica plating and immunoscreening method as previously described (Standker et al., 1997) HM7 secretes approximately twotold more mucin in culture and contains twofold more intracellular mucin compared with LS174T, whereas LM12 produces ~30% less mucin overall compared with the parental line (Standker et al., 1997). HM7 formss well differentiated tumors in nude mice, with prominent glands containing PAS-positive mucin comprising 40-50% of the

tumor area. In contrast, LM12 xenografts contain fewer mucin-containing glands comprising <10% of the tumor area (O'Reilly et al., 1997).

Cecal injection.

Confluent cultures are harvested by brief trypsinization (0.05% trypsin-0.02% EDTA in HBSS without calcium and magnesium), washed several times with calcium and magnesium-free phosphate-buffered saline (CMF), and resuspended at a final concentration of $5x10^7$ cells/ml in serum-free medium. The presence of single-cell suspensions was confirmed by phase-contrast microscopy and cell viability was determined by Trypan blue exclusion. Pathogen-free BALB/c NCR-NU athvmic mice (3-5 week old females obtained from the National Cancer Institute, Frederick, MD) were anesthetized. The cecum exteriorized, and $5x10^6$ viable tumor cells in 0.1 ml injected into the cecal wall as previously described (O'Reilly et al., 1993; O'Reilly et al., 1997). The cecum was replaced *in situ,* and the abdomen closed with stainless steel clips. After 6 weeks animals were sacrificed by cervical dislocation and the abdominal organs and thorax examined for the presence of macroscopic "primary" cecal tumors and metastases. Macroscopic metastases were defined as those visible to the naked eye. Organs including cecum, liver, mesenteric nodes, and lungs were removed and fixed in 10% formalin for 24 hours. Representative tissues were embedded in parafin and serial 5-μm sections cut, stained with hematomylin-eosin and periodic-acid-Schiff (PAS), and examined by light microscopy to verify the presence of metastases.

Splenic injection (liver colonization assay).

The ability of the various tumor cells to colonize the liver after entry into the hepatic-portal system was tested in a modification of a splenic injection model (Pennica et al., 1983; Patthy et al., 1985), as previously described (Goldhaber et al., 1988). Tumor cells were grown to confluency in 75-cm^2 tissue culture flasks, harvested as described for cecal injection, and

resuspended in serum-free DMEM at a concentration of 10^7 cells/ml. Athymic nude mice were anesthetized with methodyfluorance by inhalation, prepared sterilely, and the spleen exteriorized through a flank incision. One million cells in 100 μl were slowly injected into the splenic pulp through a 27-gauge needle over 1 minute followed by splenectomy 1 minute later. Animals sacrificed 3-4 weeks later, the livers removed and weighed and tumor nodules were counted.

The immediate fate of tumor cells after splenic injection was determined by injection of IdUR-labeled tumor cells. Cells were grown as above and 0.5 μCi/ml [^{125}I] IdUR was added to culture medium 24 hours before harvesting (Goldhaber et al., 1988). This concentration of isotope labels >95% of tumor cells without affecting viability. Cells were prepared and injected into the splenic pulp as described above. Animals were sacrificed 10 minutes after injection, and livers removed. The radioactivity present in the spleen (removed after injection), liver, and remaining organs of each animal was determined by gamma-counting in a model 7000 counter (Beckman Instruments Inc.)

Liver colonization after inhibition of mucin glycosylation.

Tumor cells were grown to 50% confluency in 7-5-cm^2 tissue culture flasks as described above. The tissue culture medium was then aspirated and replaced with fresh medium containing 2 mM benzyl-α-N-acetylgalactosamine (benzyl-α-GalNAc) (Bennett et al., 1991). Cells were harvested 48 hours later and liver colonization assayed after splenic injection as above.

In a separate experiment, tumor cells were grown for 48 hours in medium containing 2 mM benzyl-α-GalNAc as above. The cells washed three times in CMF, and the medium will be replaced with DMEM containing 10% FBS. After an additional 48 hours the cells were harvested and liver colonization assayed after splenic-portal injection. Parallel flasks were grown

for 96 hours in DMEM plus FBS or DMEM containing 2 mM benzyl-α-GalNAc (n=6 animals per group).

EXAMPLE 6

Fermentation

Growth of recombinant strain

Growth of the recombinant strain is accomplished by making five Amp/Cm plates using the following: a. 200 ml water; b. 2 g tryptone; c. 1 g yeast extract; d. 1 g NaCl; and e. 3 g agar. Next, these compounds are mixed. The pH is then adjusted to 7.4 using 2N NaOH. The mixture is then autoclaved at 120 degree C for 15 min. Then keep it in a 50 degree C water bath for 1 hour. Next, add 0.2 ml of 100mg/ml ampicillin, the ampicillin should be dissolved in water. After this, add 0.2 ml of 35 mg/ml chloramphenicol the chloramphenicol should be dissolved in ethanol. Then, put 20 ml of this in each petri dish (100 x 15 mm). Keep these at room temperature for 1 hour. Warm up 1 ml of stock solution of 3A strain at 37 degree C. After the stock solution is melted, immediately inoculate 0.05 ml of the solution on an Amp/Cm plate. Make a serial dilution. Then incubate at 37 degree C for 12-16 hours. Pick up single colony and inoculate the single colony in 2 ml LB-Amp/Cm (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, 0.1g/l ampicillin and 0.035 g/l chloramphenicol). Maintain the colony by shaking (250 rpm) at 37 degree C for 12-16 hours. Then transfer the above 2 ml culture to 100 ml LB-Amp/Cm. Maintain the shaking (250 rpm) at 37 degree C for 6-8 hours. Transfer above 100 ml culture to 10 l LB-Amp/Cm.

Fermentation

This is a case for 10 l fermentor. This can be linearly up scaled except of temperature, agitation and growth time for example: 100 l, x10; 300 l, x 30; 500 l, x 50; and 1000 l, x 100.

The first step requires dissolving 85 LB and 200 g of yeast extract in 10

l of water, this equals the components of YEEM. Next, adjust pH to 7.5 using 2 N NaOH. Autoclave the entire mixture. Keep a controlled temperature at 37 degree C. Next, begin agitation at 250 rpm. Add 1 g of ampicillin, add 0.35 g of chloramphenicol, then add 1 ml antifoam. Inoculate 100 ml of 3A culture. Maintain the agitation at 250 rpm with the pH at 7.5. Keep the air at 15 l/min/l culture. Take 0.1 ml culture into 10 ml of YEEM each 0.5 hour. It is a hundred times dilution. Read the number by photometry at 600 nm., the OD number x 100 and if "n" > 5, add 0.3 l of 50% glucose. If "m" < 6, add 0.3 l of 50% glucose. Add 0.3 l of 50% sucrose. Then add 20 ml of vitamin solution, wherein the vitamin solution includes the following: 6 g/l thiamin; 3 g/l pyridoxine; 2.7 g/l DL-pantothenic acid; 0.7 g/l pyridoxine; 0.2 g/l riboflavin; 0.03 g/l biotin; 0.02 g/l folic acid. Next, add 20 ml of metal solution. The metal solution includes the following: 75 g/l Na_3Citrate; 30 g/l $FeCl_3$; 2 g/l $CoCl_2$.; 2 g/l Na_2MoO_4; 2 g/l $CuSO_4$; 1.5 g/l $MnCl_2$; 1.5 g/l $ZnCl_2$; 1 g/l $CaCl_2$; 0.5 g/l H_3BO_3. Then begin agitation at 500 rpm, pump air at 20 l/min/l culture. Add antifoam 0.5ml/hour. When 12 < OD <15 at 600 nm, add 0.3 l of 50% glucose. When 20 < OD < 25 at 600 nm, add 0.3 l of 50% glucose.

REACTIVATION

Harvesting Cells

(Warning: Never use EDTA in following processes!) Isolate of cells from culture using membrane system. The membrane is as follows: membrane: 0.04 micron polyethersulfone; pressure: 1 to 5 psi; temperature: 4 degree C. Concentrate to 5% of the original volume. Add equal volume of PBS (10 mM sodium phosphate buffer containing 100 mM of NaCl at pH 7.5). Concentrate to 50% volume. Repeat 10 times the previous two steps. (Caution: do not get cells by centrifugation.) The cell suspension can be frozen until uses. Membrane can be regenerated in 0.1 N NaOH for 3 hours.

Cell Lysis

Use French Press method as set forth in previous examples.

Obtain Inclusion Bodies

Centrifugation is utilized for obtaining inclusion bodies. Maintain a temperature of 4 degree C. Centrifuge at 10000 x g for 30 min. Keep the pellets, because the pellets are crude product, and the pellets are called as "KED inclusion bodies". Do not measure the protein concentration of inclusion bodies. Usually, measurement of protein concentration of inclusion bodies is under estimated. Measure the wet weight of inclusion bodies. Freeze the inclusion bodies (crude product) in -80 degree C.

Purify Inclusion Bodies

Take the inclution bodies of KED from the -80 degree C freezer. Put liquid nitrogen on the crude product. Suspend the crude product in PBS (0 mM sodium phosphate buffer containing 100 mM of NaCl at pH 7.5). Then adjust the concentration of inclusion bodies at 0.1 to 0.25 g wet weight / ml PBS. Add Tween-20 to a final concentration of 0.1% and agitate for 1 hour at 4 degree C. A single pass through the French Press Cells is all that is required. Add pre-cooled (4 degree C) PBS the ten times of original volume.

Concentration of the inclusion bodies is determined using the membrane system as follows: membrane: 0.1 micron polyethersulfone; pressure: 1 to 5 psi; and temperature: 4 degree C. Then concentrate to 10% of the original volume. Add Tris buffer (100 mM Tris at pH 8.5) to bring the volume to the original. Repeat the above steps five times. Obtain final concentration of inclusion bodies 0.1 to 0.25 wet weight / ml Tris buffer. Keep the inclusion bodies in the Tris buffer at -20 degree C as needed.

Solublization of Inclusion Bodies

For the mild solubilization of KED protein, prepare a small fermentor 1 to 2 little. Add 0.75 l of solubilization solution. Add the components of solubilization solution (SS).

Components	Per little	Concentration
Urea	480 g	8 M
Tween-20	1 ml	0.1%
Tris	12.1 g	0.1 M

Next bring the pH to 8.5, using concentrate HCl, be careful not to use more than 0.5 l of water. Add 100 ml of crude product (inclusion bodies). Then agitate at 50 rpm. Add 1 ml of beta-mercaptoethanol. The final concentration of beta-mercaptoethanol should be 140 mM. Keep at 25 degree C for 0.5 hours. Measure the protein concentration. Control should be the SS containing 140 mM beta mercaptoethanol. Adjust the volume to 20 mg proteins / ml by SS containing 140 mM mercaptoethanol. Agitate at 50 rpm for 2.5 hours.

Refolding

Follow the refolding steps set forth in the previous examples and the composition as follows:

Components	**Amount/l**	**Concentration**
Tris	12.1 g/l	100 mM
L-arginine	160 g/l	0.8 M
Tween-20	1 ml	0.1%
GSH	300 mg	0.9 mM
GSSG	120 mg	0.19 mM
Zinc Chloride	1.36 mg	0.01 mM
3A	100 mg	0.003 mM
Temperature	RT	
Time	20 to 24 hrs	
pH	8.8 with HCl.	

PURIFICATION

Heparin-Sepharose Chromatography

Twenty ml of dialyzed KED protein solution was diluted in 180 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl. The protein

solution was loaded on a Heparin-Sepharose column (HiTrap pre-packaged 5 ml column, Pharmacia, Piscataway, NJ). Columns were washed with the same buffer until the OD_{280} returned to the base line. KED protein was eluted by a NaCl gradient (150-1000 mM). Pure KED protein eluted within 210-330 mM NaCl was identified by 10% polyacrylamide gel electrophoresis.

The following is a typical process using "Hi-Trap Heparin-Sepharose": Twenty ml of refolded KED protein solution was diluted in 180 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl. The protein solution was loaded on a Heparin-Sepharose column (HiTrap pre-packaged 5 ml column, Pharacia, Piscataway, NJ). Column was washed with the same buffer until the OD_{280} backs to the base line. KED protein was eluted by a NaCl gradient (15-1000 mM). Pure KED protein eluted within 195-345 mM NaCl was identified by 10% polyacryl amide gel electrophoresis.

Acticlean Etox Chromatography

Acticlean Etox (Sterogene, Arcadia, CA) resins were cleaned with 1 M NaOH for 12 hours at 4 °C, and packed to column. The column was washed with PBS until pH below 7.6. Pure KED protein was dialyzed against PBS, and loaded on Actilean Etox column. Each ml of Acticleas Etox gel was used for treatment 5 mg of KED protein. The KED protein was then sterilized by passing a 0.2 microm filter.

QUALITY CONTROL

Polyacrylamide Gel Electrophoresis

Follow the polyacrylamide gel electrophoresis steps as set forth in the above examples.

Immunological Detection

Cut two pieces of Whatman paper to cover the size of the gel with moistened transfer buffer. Place one piece of nitromembrane filter on the gel.

Transfer proteins on to nitromembrane, then transfer buffer containing the following: 20% methanol; 0.025 M Tris; 0.19 M glycerol; and having pH = 8.5. Transfer volts equalling 25. The transfer time is >3 hours with a transfer temperature of 4 degree C. Alternatively the transfer volts can equal 100, with a transfer time of 1 hour and a transfer temperature RT. An incubation blot in PMT, wherein PMT includes the following: PBS containing 5% non-fat milk & 0.05% Tween-20 with an incubation time of 1 hour and an incubation temperature at 37 degree C. Then incubate with rabbit anti recombinant KED IgG at a concentration of 1500 to 2000 times. The incubation time should equal 1 hour with an incubation temperature of 37 degree C. Wash three times with PMT, each wash should be for 10 minutesat 37 degree C. Then incubate with rabbit anti-IgG antibodies 1: 2000. The anti-IgG is peroxidase conjugated and incubation time is equal to 1 hour with an incubation temperature of 37 degree C. Again wash three times with PMT as before. Then sash with PBS. Next incubate with the following: 10 mg 4-chloro-1-naphthol; 3 ml of methanol; 17 ml of PBS; 0.02 ml H_2O_2 with a reaction temperature of 37 degree C and a reaction time of 5 to 30 min. Next add a stop solution of 0.05 ml 3 M NaOH.

Endotoxin Control

Harvesting of cells by membrane includes the following: membrane: 0.04 micron polyethersulfone at a pressure of 1 to 5 psi and a temperature of 4 degree C. Then wash cells by PBS (10 mM sodium phosphate buffer containing 100 mM of NaCl at pH 7.5). Repeat washing 3 times. Repeat washing reduces endotoxin contents in the final products. Endotoxin secreted in medium from cells can be excluded by membrane system. Regenerate membrane in 0.1 N NaOH for 3 hours.

Stage of inclusion bodies

Method I: Centrifugation occurs at a temperature of 4 degree C, at a speed of 10000 x g for 30 min. Then incubate the pellets (crude product) in

liquid nitrogen for 10 min. Suspend the crude product in PBS (10 mM sodium phosphate buffer containing 100 mM of NaCl at pH 7.5). Next, adjust the concentration of inclusion bodies at 0.1 to 0.25 g wet weight / ml PBS.

Method II: Extraction occurs by adding Tween-20 to a final concentration of 0.1%. Then agitating for 1 hour at a temperature of 4 degree C.

Method III: French Press Cells in a single pass.

Method IV: The membrane system involves adding pre-cooled (4 degree C) PBS the ten times of original volume. The membrane is as follows: 0.1 micron polyethersulfone at a pressure of 1 to 5 psi with a temperature of 4 degree C. Then wash with PBS buffer.

Stage of purification

Use Acticlean Chromatography as set forth previously, because it has a high affinity with endotoxin and it reduces endotoxin contents 10 to1000-folds.

Material Qualification

	Materials	**Comments**
1.	Na_2HPO_4	Grade I
2.	$(NH_4)_2SO_4$	Grade I
3.	$MgSO_4$	Grade I
4.	$FeCl_3$	Grade I
5.	$CoCl_2$	Grade I
6.	Tris-HCl	Grade I
7.	$NaMoO_4$	Grade I
8.	$CuSO_4$	Grade I

9.	$MnCl_2$	Grade I
10.	$ZnCl_2$	Grade I
11.	$CaCl_2$	Grade I
12.	H_3BO_3	Grade I
13.	Na_3citrate	Grade I
14.	Thiamin	4 degree C / half year
15.	Niacin	4 degree C / half year
16.	Pantochenic Acid	4 degree C / half year
17.	Pyridoxine	4 degree C / half year
18.	Riboflavin	4 degree C / half year
19.	Biotin	4 degree C / half year
20.	Folic Acid	4 degree C / half year
21.	Tryptone	No moisture
22.	Yeast Extract	No moisture
23.	Glucose	Stable
24.	LB Broth	No moisture
25.	Reduced Glutathione (GSH)	Fresh, no moisture
26.	Oxidized Glutathione (GSSG)	Fresh, no moisture
27.	Urea	Powder, no aggregation
28.	beta-Mecaptoethanol	Tightly capped
29.	Tris-HCl	Grade I
30.	HCl	Keep in hood
31.	NaOH	No moisture
32.	Na Acetate	Grade I
33.	Acetic Acid	Stable
34.	Tween-20	Stable
35.	Ampicillin	4 degree C / half year
36.	Chloramphenicol	-20 degree C / 5 years
37.	Glycerol	Stable
38.	Maltose	Stable
39.	Agarose	Stable

40.	Isopropyl-beta-D-Thiogalactopyranoside (IPTG)	-20 degree C / 2 years
41.	NaCl	Stable
42.	L-Arginine Hydrochloride	Stable
43.	SP-Sepharose	0.1 M NaCl / 25% ethanol
44.	Heprin-Sepharose	0.1 M NaCl / 25% ethanol.
45.	Acticlean	4 degree C
51.	Antifoam	Stable
52.	D-Val-Leu-Lys-pNA	-20 degree C
53.	Glu-Plasminogen	-80 degree C
54.	CNBr-Fibrinogen Fragments	-80 degree C
55.	GLC-1000 Chromogenic LAL	4 degree C
56.	Thrombin	-80 degree C
57.	Fibrinogen	-80 degree C

PRE-CLINIC

Assay Method

Endothelial culture Bovine capillary endothelial cells (BCE cells) were obtained for the proliferation assay, cells were washed with PBS and dispersed in a 0.05% trypsin solution. A cell suspension (25,000 cells/ml) was made with DMEM + 10% BCS and plated on 24-well culture plates (0.5 ml/well) and incubated at 37 degree C in 10% CO2 for 24hr. The medium was replaced with 0.5ml DMEM +5% BCS + 3ng/ml recombinant bFGF and the test sample applied. After 72hr, cells were dispersed in trypsin, resuspended and counted using a Coulter counter. Inhibitory activity of test compounds was expressed as a percentage inhibition of endothelial cell growth in culture compared to endothelial cells in the absence of the test compound.

To determine the ability of the test compounds to block migration of endothelial cells toward the angiogenic factor bFGF, migration assays were performed in a modified Boyden chamber using BCE cells. Cells were grown

in DMEM supplemented with 10% FBS and supplements at passage 15. To assess migration, the cells were starved overnight in DMEM supplemented with 0.1% BSA, harvested, suspended in DMEM/BSA, plated at 10^6 cells/ml on the lower surface of a gelatinized membrane (Nucleopore Corp., Plesanton, CA) in an inverted Boyden chamber and incubated for 1.5 hours to allow cell attachment. The chambers were then righted, test material was added to the top well and incubated for 5 hours. Membranes were then fixed and stained and the number of cells migrating to the top of the filter in 10 high-powered fields were counted. DMEM with 0.1% BSA was used as a negative control and bFGF at 10 ng/ml was used as a positive control.

Assay Results

Proliferation assay using bovine endothelial cells indicated that fusion protein KED completely inhibited endothelial cells migration at concentration as low as 8 nM (250 ng/ml). Where, human angiostatin and endostatin show almost no activity at 12 nM (250 ng/ml) by the same assay. The observed IC_{50} of KED is less than 100 ng/ml. IC_{50} of human endostatin in the assay was 350 ng/ml. These results indicated that the human fusion protein KED is much stronger than endostatin, the strongest anti-angiogenic agent reported to date.

CORNEAL ASSAY

The corneal assay was performed. 5 uL hydron pellets (Hydron Laboratories, New Brunswick, NJ) containing 50 ng bFGF or 5 ng KED protein and 10 ug/ml sulcrafate were implanted in a corneal pocket of anesthetized rats. After 7 days, the animals were sacrified, perfused with colloidal carbon, and the excised corneas were mounted for microscopy analysis.

The rats are anesthetized with Nembutal (29mg/kg body weight) being sure carefully clip all whiskers around the snout. Next, position the animal

under the dissecting microscope, proptose the eye and secure in place with a hemostat. Irrigate the cornea with Ringers (disclosed below) to remove any hair or debris. Make a 2mm incision into the cornea at a point just slightly off the center of the cornea using the tip of a No. 11 Bard Parker blade. Using the tip of the scalpel blade, position the tip in the center of the cornea and make a 1-2mm incision into but not through the cornea. When viewed under the dissecting scope you should just a slight separation/ effacement of the corneal surface revealing the underlying stroma. A single incision is all that is needed.

Once the corneal stroma is entered, take an iris spatula, place the tip under the lip of the incision and begin to gently but firmly blunt dissect in a slightly downward direction, through the stroma to a point approximately 1-1.5mm from the limbus. If necessary bend the iris spatula so it has a slight downward curve to it. Avoid getting too close to limbal vessels. This is the most frequent cause of non-inflammatory, false positive responses. Moisten one of Hydron pellets and using curved Dumont forceps place it near the opening of the pocket and under the scope inset it into the top of the pocket. Use your spatula to gently force the pellet down to the base of the pocket. The pellet should occupy not more than one-half the depth of the pocket. Anything larger will be extruded due to intraoccular pressure and will prevent the opening of the pocket from sealing spontaneously.

When the implant is in position, flush the eye with saline and proceed to the next eye. It is important to work as quickly as possible to avoid prolonged heating of the eye by the microscope lamp. It is advisable to get a cold light source if possible. Examine the corneas every other day and sacrifice animals that show any evidence of corneal exudation. Some corneal edema is expected within the first 24 to 36 hours. This should not persist after this time, however. Responses are usually fully developed within 5 to 7 days. Dissect through the stroma by rocking the spatula laterally back and

forth. Continue dissecting through the stroma until you are approximately 1mm from the limbus. Irrigate the eye thoroughly with Ringers then pick up a hydrated pellet (Hydron or ethylene/vinyl acetate) with the forceps and insert into the pocket. Force it down to the base of the pocket using the spatula. Re-irrigate the eye. Ideally, the implant should occupy no more than three-fourths of the pocket. Anything larger will be extruded or the pocket will fail to seal.

When the procedure is completed, remove the hemostat and gently reposition the eye in the socket. Avoid touching the pocket. Except for some transient limbal and iris vasodiatation, the eyes will appear entirely normal after 24 hours. Examine eyes as usual for presence of developing capillary sprouts. Any corneal edema that persists for more than two days likely indicates inflammation.

Lactated Ringers

Each 100 ml of solution contains: 600 mg of Sodium chloride; 310 mg of Sodium lactate: 4.61 ml of 60% solution; 30 mg Potassium chloride; 20 mg calcium chloride; and has a final ph 6.5 - 7.0. Sodium lactate is supplied as a 60% syrup solution: FW = 112.1 g/liter, thus 6.726 g sodium lactate in a 60% solution, 4.61 ml of a 60% sodium lactate solution is 310 mg of sodium lactate. Store this at 4°C. Add sodium heparin at a final concentration of 5U/ml for perfusion. Prepare at least 2 liters of the above solution, because approximately 100-200 ml are needed for perfusion of a single 100-200 g rat.

PROCEDURES FOR PREPARATION OF HYDRON

Preparation of Hydron:The Hydron is supplied in the form of a fine powder. Make a 12% solution; (ordinarily 1 gm lots in 8 mls or 3 gm lots in 25 mls), in absolute alcohol (pure ethyl alcohol USP). To dissolve, leave at 37°C for 24 hours in a rolling apparatus (e.g., Forma-Scientific CEL-roll) or a shaker at low speed. No preservative is necessary. Store at room temperature.

Incorporation of Proteins in Polymer:

Method I (for lyophilized materials):

The desired weight of lyophilized protein is added to the hydron in solution and mixed in a vortex mixer. To prepare pellets for insertion in the guinea pig cornea, aspirate 5 μl in a micropipette and drop onto a sterile glass slide. For other purposes, various amounts of the polymer can be pipetted into wells or troughs of the desired shape and size. Discs are good for release studies).

Dry in a dessicator overnight. For the corneal pellets, the slides are placed in sterile petri dishes overnight in a laminar flow hood.

Rehydrate by placing a drop of sterile lactated Ringer's solution on the pellet or in the trough. The hydron will swell up to a certain extent. Wait for at least 5 minutes, when most of the rehydration has occurred. The pellets can then be pulled with a tweezer or a fine spatula. Note the release starts virtually immediately on rehydration. Thus, if one needs to quantitate, zero time must be consistent.

Method II:

If the protein to be used is in solution, pipette the desired quantity of aqueous solution over the alcoholic hydron. Do not use more than 6 parts of aqueous solution to 4 parts of hydron; otherwise, the hydron will precipitate. For the corneas, one-to-one ratios of conditioned media and hydron are used. Mix in vortex, then will well, and put on slides or troughs as explained in Method I. Note that with this method, one ends up with less hydron, thus faster release.

Methods of Changing Release Times:

A. The more hydron, the slower the percent release.

B. Triple sandwich (3-day procedure)

Pipette desired μl's of hydron (without protein) onto a glass slide or trough. Dry overnight. Add to dried pellet, a middle layer of hydron plus protein. Dry overnight. Top it off with hydron without protein. Dry overnight.

C. "Sloppy" sandwich (2-day procedure):

Pipette hydron with protein onto a glass slide, dry. Remove pellet with tweezers and dip into a puddle of hydron. Dry.

Maintain sterile and clean conditions, especially if material is to be tested in vivo. Most of the procedures are performed in a tissue culture hood, using sterile kits, which have spatulas, tweezers, etc. Hydron is much less sticky than Elvas, and can be handled more easily.

With some batches, and if a large pellet is to be dried, bubbles can form during the drying procedure, so it might be necessary to dry in a vacuum oven (equipped with millipore filter). This has not been necessary with the small pellets used in the experiments.

The mechanism of release from the polymer is unknown. Hydron is neutral, but with the small numbers of compounds tested, it appears that both negatively and positively charged proteins are released to the same extent. There are also differences in the kinetics of release, dependent on molecular weight, but these are not tremendous.

ANIMAL MODEL

Efficacy of KED in Glioma Model

Using validated culture and implantation techniques, nude mouse harboring human glioma (U251MGn) were treated with KED for a period of 14-21 days weeks beginning 1 week following implantation. Beginning 2

weeks following implantation, 8 animals in each of the control and treatment groups were undergo MR imaging and were sacrificed for histochemical and *in situ* detection protocols for quantitation and to allow registration of MR imaging with molecular histology. Subsequent groups of 8 animals in control and treatment groups were sacrificed weekly following neuroimaging for a total of 4 sessions, a total of 6 weeks following implantation. A randomized cohort of animals implanted had been treated and survival studies undertaken. Results have been fully duplicated in a repeat set of animals with a second human glioma cell line (U87MG).

Cell Lines

U251MGn:

This cancer cell line was originated by Pónten from a patient with a glioblastoma. The culture was initially mycoplasma positive was cured by antibiotic treatment in culture.

U87MG:

The cell line was initiated from an anaplastic astrocytoma.

Culture:

All cell lines are routinely mycoplasma tested and have tested free of contamination. Cells are routinely maintained in DMEM + 10% FCS in a 37°C humidified incubator in 10% CO_2.

Cancer Implantation

Following guidelines in an approved animal use protocol, nude mouse were inoculated with cancers as follows.

Preparation of Cancer Cells:

Cells were harvested from 90% confluent culture plates by treatment with a 0.1% trypsin/EDTA for 10 minu. Cells were rinsed off the plates with

media and spun for 10 min. at 1000 rpm. The cell pellet was resuspended in 100 :L of culture media, counted and the concentration of cells adjusted to $5x10^5/5$:l. Viability of the cells was tested using Trypan blue exclusion counting on a Hausser Scientific Brightline hemocytometer.

Implantation:

A #2701 10 :L Hamilton syringe with a #4 point, 26s gauge needle hold tumor cell suspension [500,000 viable cells]. The cell suspension was injected stepwise at a rate of 0.5 :L/10 seconds until the entire volume had been injected. During and after the injection, careful note was made of any reflux from the injection site. Animals were maintained for 1 week prior to treatment.

KED Treatment

This was a two-sample, placebo-controlled trial in tumor-bearing mouse of KED TREATMENT, where the placebo was saline, the drug group was suspension solution, the vehicle for administration of KED. The primary endpointis tumor volume, the margins measured as the infiltration border. There were two complete replications of the trial for each cell line, U251MGn and U87MG. For each replication, 4 nude rats were inoculated with tumor cells. After one week of maintenance, half the rats were randomly treated with KED and half were received saline.

Description of the animal model:

The prognosis for patients with malignant gliomas has not significantly changed in recent years. Despite debulking surgery, radiation and cytotoxic chemotherapy, progression is the rule and median survival is still measured in weeks. These tumors have mortality ratio of 77%. Brain tumors constitute the second leading cause of cancer deaths in males and females under age 15, third for adult males and the fourth cause for females 15-34 years. In the 35-54 year age group, brain tumors remain the fourth

leading cause of cancer deaths in males. Thus, brain tumors are highly fatal and often strike patients in their most productive years. Finally, the incidence of glioblastoma multiforme (GBM) is also rising, especially in older adults, the poorest prognostic group. Rapid development and assessment of novel approaches to therapy are required for this incurable malignancy.

The patterns of growth and recurrence by infiltration in these lesions confounds local approaches to therapy, such as surgery and radiation therapy. This regional infiltration during tumor progression has been most strikingly shown in the whole-mount where these tumors display a central area of necrosis, a highly cellular rim of tumor and a peripheral zone of infiltrating cells. Infiltration occurs along white matter tracts, around nerve cells, along blood vessels and beneath the pia (secondary structures of Scherer). Studies have shown that tumor cells have migrated from the primary site of malignant gliomas by the time of diagnosis in the majority of cases and are responsible for the inevitable local recurrence and tumor progression seen clinically . Recurrence of human gliomas following surgery and radiation is most commonly seen in the margin adjacent to the initial tumor but can also be remote. This infiltration zone, from which most local and regional recurrences arise, is all but invisible using conventional imaging techniques.

Gliomas in general, and more anaplastic gliomas in particular, infiltrate and spread great distances in the brain. As a result, despite aggressive surgery, radiation and adjuvant chemotherapy, few durable responses are achieved and survival continues to be measured in weeks. In histological sections, most glioblastomas contain a central area of necrosis, a highly cellular rim of tumor and a peripheral zone of infiltrating cells. Infiltration occurs along white matter tracts, around nerve cells, along blood vessels and beneath the pia (secondary structures of Scherer). Elegant studies have shown that tumor cells have migrated from the primary site of malignant

gliomas by the time of diagnosis in the majority of cases and are responsible for the almost inevitable local recurrence and tumor progression seen clinically. Recurrence of human gliomas following surgery and radiation is most commonly seen in the margin adjacent to the initial tumor but can also be remote. Clearly more effective treatment approaches are required to improve local tumor control in order to prevent recurrence, progression and improve survival beyond that achievable today.

Recently, the concept of cytostatics being used to restrain tumor progression (rather than induce cyto-reduction as conventional cytotoxic chemotherapies intend) has emerged. This concept questions the current therapeutic model in cancer management, derived from microbiology, in which cancer cells are considered to be different from the host, and these differences exploited therapeutically. Continuing the analogy to infection, conventional wisdom has purported that unless cells are killed and totally eliminated, they will overwhelm the host. Research strategies, new drug development, and measures of therapeutic success have been based on this killing paradigm. A regulatory model has recently been proposed in which cancer is viewed as a dynamic maladaptive process that originates within the host, is constantly in evolution, and is potentially reversible. This model is consistent with the molecular genetic understanding of cancer processes such as clonal evolution as was demonstrated in gliomas and offers a provocative reinterpretation of long-held clinical and laboratory observations. One of the implications of such a model is that, by reimposing biological control on a cell population, functional control of a tumor can be gained and this can not require complete tumor elimination. In fact, not every cancer cell need be killed to achieve control, according to this model. Cancer is a process characterized by growth, invasion, and angiogenesis, all of which have their equivalents in normal tissues. Management of these components of the malignant phenotype constitute a novel avenue for therapeutic research. Conventional antineoplastic approaches such as surgery, radiation

therapy, and cytotoxic chemotherapy play roles as debulking modalities in the regulatory model, whereas biologic strategies are intended to induce re-regulation and long-term tumor control. Anti-invasion therapy represents one of these strategies in malignant gliomas and rest on a molecular understanding of the process.

The process of glial tumor invasion has been studied *in vitro* for some time, but the molecular pathophysiology has not been clearly addressed. It is presumed that disordered proteolysis, adhesion, motility and angiogenesis are involved in glial tumor invasion as they appear to be in systemic cancers. The results with cysteine proteases, for example, clearly demonstrate that cathepsin B (CB) is expressed in glial tumor cells, in amounts related to the degree of malignancy. CB is seen in the infiltrating tumor cells and in the proliferative neovascular endothelium of the infiltrating margin, compatible with the hypothesis that CB is functional in the processes of tumor invasion and angiogenesis. Specific inhibitors of cathepsin B have been developed as potential anti-invasion agents in gliomas and appear active in the suppression of invasion in model systems *in vitro*. Many studies have also shown a role for the type IV collagenases in tumor cell invasion, angiogenesis and metastasis and also in glioma growth and invasion. Matrix metalloprotease-2 (MMP-2) *in vitro* has been subcellularly localized to invadopodia. Inhibition of these enzymes by endogenous tissue inhibitors of metalloproteinases (TIMPs) has shown inhibition of tumor cell invasion in experimental models. Clinical anti-invasive agents for MMP inhibition have also been developed, such as BB94 and BB-2516.Completing the list of proteases in glioma, the plasminogen activator system also appears to be up-regulated, particularly in malignant gliomas. Finally, the acquisition of cellular motility and angiogenesis are felt to be central to the process of glioma invasion. Cell adhesion molecule and extracellular matrix alterations have been described in glioma model systems and, interestingly, laminin, a molecule not normally present in the brain except at the glia limitans and in the perivascular space, is upregulated in the

presence of invading glioma cells, produced by surrounding normal astrocytes. Other factors, such as the autocrine motility factor Autotaxin and Glioma-Derived Motility Factor, are expressed in malignant gliomas *in vitro*. Finally, VEGF, bFGF and TGF are expressed locally during the process of glioma angiogenesis, where the branching proliferation of endothelial cells is preceded by proteolysis, and endothelial migration.

EXAMPLE 7

Materials and Methods

1. Cloning of tPA Kringles

Human fetal brain mRNA was used tor isolation of tPA kringle domains. Reverse transcription was carried out in 20 μl reaction solution containing 5 μg of human fetal brain mRNA (Clontech), 250 ng of oligo $(dT)_{17}$ primer, 5 mM DTT, 2.5 mM MgCl, 450 μM of dNTP and 200 units of reverse transcriptase (BRL) in 50 mM Tris buffer (pH 8.3). Briefly, reaction mixture without reverse transcriptase was heated at 72°C for 10 minutes to denature mRNA and was quickly chilled on ice for 15 minutes. After 5 minutes incubation of the contents at 42°C, an hour reaction was initiated by adding of reverse transcriptase. The resulted cDNA from above reaction mixture were used for amplification of tPA kringles by using a proofreading thermostable Pfu DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction solution (100 μl) contains 20 mM Tris (pH 8.0), 2 mM MgCl, 10 mM KCl, 0.1% Triton X-100, 5 μl of cDNA, 150 ng of each primer and 7.5 units of Pfu DNA. The PCR process was initiated by "hot start" (Ault et al., 1994). Twenty-five reaction cycles consist of 45 seconds at 94°C for denaturing, 45 seconds at 59°C for annealing and 3 minutes at 68°C for DNA synthesis. Kringle 1 was amplified by a 5'-end primer of GGAATTC-[NdeI]-ATAGATACCAGGGCCACGTGCTACG, and a 3'-end primer of CCG-[BamHI]-TTAGTTTCCCTCAGAGCAGGCAGG. Kringle 2 was amplified by a set of primers: GGAATTC-[NdeI]-AACAGTGACTGCTACTTTGGG for 5'-end and

CCG-[BamHI]-TTAGGTGGAGCAGGAGGGCACATC for 3'-end. A DNA fragment containing both of kringles was also amplified by a 5'-end primer of GGAATTC-[NdeI]-ATAGATACCAGGGCCACGTGCTACG, and a 3'-end primer of CCG-[BamHI]-TTAGGTGGAGCAGGAGGGCACATC. Where, GGAATTC-[NdeI]- and CCG-[BamHI]- are adaptors containing NdeI and BamHI recognition sites.

The PCR products containing the tPA kringle sequences were purified through the PCR Select-II column (5 Prime-3 Prime, Boulder, CO). The purified DNA fragments were digested by restriction enzymes of NdeI and BamHI at 37°C for 2 hours. The digested DNA fragments with NdeI and BamHI cohesive ends were purified by phenol extraction, ethanol precipitated and used to insert into pET11a vector. The pET11a vector was linearized by the same enzymes of NdeI and BamHI. The linearized vector was further treated by calf intestinal alkaline phosphatase (CIAP) to prevent self-ligation of vector inter or intra molecule. The dephosphorylated vector was ligated with tPA kringle fragments. The ligation solution was used to transform competent *E. coli* TG1 cells. Recombinant plasmids were analyzed by double digestion of NdeI and BamHI enzyme. Recombinant plasmids were confirmed by DNA sequencing of dideoxy chain termination (Sanger et al., 1977).

2. *PCR-Mediated Mutagenesis*

Mutagenesis was carried out using recombinant plasmid $pK2_{tPA}$ containing the wild type tPA kringle 2 cDNA. A PCR-mediated mutation kit, Quick-Change (Stratagene, La Jolla, CA) with some modification was employed for the process. Fifty μl PCR reaction mixture was prepared on ice. The mixture contains 20 mM Tris (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM Mg SO_4, 0.1% Triton X-100, 100 ng pure super-coiled $pK2_{tPA}$ DNA, 200 ng each of sense and complementary mutation oligonucleotide primers, 200 μM of dNTP and 5 units of cloned Ptu DNA polymerase. A control PCR

reaction was simultaneously included in the thermal cycles by missing of both of sense and complementary mutation primers. Each PCR cycle consisted of denaturation at 95°C for 0.5 minutes, annealing at 55°C for 0.5 minutes, and extension at 68°C for 18 minutes, for a total of 12 cycles.

Oligonucleotide primers used for mutation PCR are listed. The mutation sites are underlined. Residue numbers are according to the amino acid sequence of tPA.

S mutant (Ser186 - Lys mutation):

SKf-1, sense, 5'-GCTACTTTGGGAATGGGAAAGCCTACCGTGGC-3'

SKr-2, anti-sense, 5'-GCCACGGTAGGCTTTCCCATTCCCAAAGTAGC-3'

Y mutant (Tyr214 - Phe mutation):

YFf-3, sense, 5'-CCTGATAGGCAAGGTTCACAGCACAGAACCCC-3'

YFr-4, anti-sense, 5'-GGGGTTCTGTGCTGTGAAAACCTTGCCTATCAGG-3'

N mutant (Asn218 - Thr mutation):

NTf-5, sense, 5'-GTTTACACAGCACAGACCCCCAGTGCCCAGGC-3'

NTr-6, anti-sense, 5'-GCCTGGGCACTGGGGGTCTGTGCTGTGTAAAC-3'

G mutant (Gly225 - Glu mutation):

GEf-7, sense, 5'-GTGCCCAGGCACTGGAACTGGGCAAACATAAT-3'

GEr-8, anti-sense, 5'-ATTATGTTTGCCCAGTTCCAGTGCCTGGGCAC-3'

K mutant (Lys240 - Gly-Gly mutation):

KGGf-9, sense, 5'-CCTGATGGGGATGCCGGTGGCCCCTGGTGCCACG-3'

KGGf-10, anti-sense, 5'-

CGTGGCACCAGGGGCCACCGGCATCCCCATCAGG-3'

H mutant (His244 -Tyr mutation):

HYf-11, sense, 5'-GCCAAGCCCTGGTGCTATGTGCTGAAGAACCGC-3'

HYr-12, anti-sense, 5'-GCGGTTCTTCAGCACATAGCACCAGGGCTTGGC-3'

W mutant (Trp253-Glu254 -Tyr-Asp mutation):

WEYDf-13, sense, 5'-CCGCAGGCTGACGT ATGATTACTGTGATGTGCCC

WEYDr-14, anti-sense, 5'-

GGGCACATCACAGTAATCATACGTCAGCCTGCGG

A 25 μl aliquot from the resulted reaction mixture was taken for restriction digestion with 10 units DpnI at 37°C for 30 minutes. Two μl of digested solution mixture was used for transformation in *E. coli* TG1 cells. Ampicillin resistance plasmids were isolated from the TG1 cells. Confirmation of mutation was performed by dideoxynucleotide DNA sequencing.

3. *E.xpression of tPA kringles*

The purified recombinant plasmids were used to transform expression strain of *E. coli* BL21 (DE3) cells by the standard $CaCl_2$ procedure. *E. coli* BL21 (DE3) cells were co-transformed with a low-copy plasmid pDC952, a derivative of pACYC184 containing argU gene. Ten colonies with ampicillin and chloramphenicol resistance for each construct were selected for gene expression analysis. Each single colony was inoculated in 2 ml of LB medium containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol. After 18 hours incubation at 37°C, 0.1 ml culture from each sample was diluted into pre-warmed (37°C) 10 ml LB, and continued growth was facilitated with shaking of 250 rpm. Protein expression was induced at the early log phase between 0.4 to 0.6 OD at 600 nM by adding of IPTG (isopropyl β-D-thiogalactopyranoside) to the final concentration of 0.5 nM. After 2 hours induction, the cultures were chilled on ice and centrifuged at 4°C. The resulting cell pellets were washed once by PBS (phosphate saline buffer) and stored at -20°C until use.

Large scale expression of protein was carried out using a fermentor (Bioflo2000, New Brunswick Scientific, New Brunswick, NJ). The injection of air to the fermentor was maintained at 15:1 air/1 culture/minute. The medium used in fermentor was LB enriched with 35 mg/ml yeast extract. Anti-foam agent 289 (Sigma, St. Louis, MO) was added to the medium at the concentration of 0.005% (v/v) to prevent foam-formation during fermentation. Protein induction was performed by 1 mM IPTG at 0.5 OD (600 nM). After 2 hours induction, EDTA (ethylenediaminetetraacetic acid) solution was added

into fermentor at final concentration of 5mM. Cells were collected by filtration system using 0.04 μrn polyethersulfone membrane (North Carolina SRT, Cary, NC).

Cells expressing tPA kringles were washed by ice-cold PBS solution three times in order to remove endotoxin secreted by the *E. coli* cells. The washed cells were re-suspended in PBS solution containing 1 mM EDTA, flowed by passing through a French Press Cells (SLM Instruments, Urbana, IL) twice at 20,000 psi. The inclusion bodies were collected by centrifugation of the cell lysate by 10,000 g at 4°C for 20 minutes. The inclusion bodies were washed three times by ice-cold PBS containing 0.5 NP-40 detergent (Sigma, St. Louis, MO).

4. *Sephadex G-75 Chromatography*

Super-fine Sephadex G-75 (Pharmacia) of 10 g was suspended in 100 mM Tris (pH 8.6) containing 1 mM EDTA and 8 M urea at 37°C for 24 hours. The resins were packed on several columns (250 nM x 15 nM). The columns were kept at 28-30°C for preventing the precipitation of urea. Inclusion bodies were dissolved in l00 mM Tris (pH 8.6) containing 8 M urea. 1 mM EDTA, 0.1% Tween-20 detergent, 145 mM of β-mercaptoethanol at the concentration of 10 mg/ml of proteins. The solution was incubated at 37°C for 1 hour and loaded (5 ml) on the above Sephadex G-75 column equilibrated by 100 mM Tris (pH 8.6).and 8 M urea. The reduced kringles were collected from the last peak from the column.

5. *Refolding Kringles*

The reduced tPA kringles were added into refolding solution containing 100 mM Tris (pH 8.8), 1 mM EDTA, 0.1% Tween-20, 0.5 M L-arginine, 1 mM of reduced gluthionine (GSH) and 0.1 mM oxidized gluthionine (GSSG). The protein concentration in the refolding solution was adjusted to 100 mg/l. The refolding reaction was performed at 23 °C for 24 hours.

6. *Lysine-Agarose Affinity Chromatography*

The refolded protein solution was concentrated by using an ultra-filtration system consisting of TRIPORT Module/316L Stainless Plates with 0.1 m^2 working surtace and a regenerated cellulose membrane molecular of 5 kDa weight cut-off (North Carolina SRT). The filtration was performed at pressures of 25 to 30 psi. The concentrated protein solution was diluted into 100 mM Tris (pH 8.0) pre-cold at 4°C to a final concentration of 25 mM of L-arginine. The diluted protein solution was then applied on a lysine-agarose column pre-equilibrated with the same 100 mMTris at pH 8.0. Approximately 1 mg protein was loaded per one ml of lysine-agarose gel. The column was continued by washing using the same buffer until OD of eluant declines to zero at 280 nM. The tPA kringles were eluted from the column by a strong lysine competitor of 6-amino-n-caproic acid using a gradient from zero to 200 mM. Each traction eluted trom lysine column was monitored at 280 nM. Samples from each peak were analyzed by 16% SDS-PAGE

7. *Acticlean-Etox Chromatography*

The pooled pure recombinant tPA kringles were dialyzed against 1000 times of PBS (v/v). Since *E. coli* cells secrete endotoxin, preventing contamination of tPA kringles from endotoxin was performed by using Acticlean-Etox (Sterogene, Arcadia, CA) chromatography. Acticlean-Etox resins were previously washed by 1 M NaOH for 12 hours at 4°C and packed into column. The column was washed using PBS until pH was below 7.6 before passing the recombinant tPA kringles. One ml of Acticlean-Etox gel was used for every 10 mg of tPA kringles. Finally, the recombinant kringles were sterilized by a 0.2 μm filter.

8. *Protein.analysis*

Protein concentration was determined by a protein assay kit (Bio-Rad Laboratories). Protein samples were prepared by heating to 100°C for 10

minutes in SDS sample buffer. SDS-polyacryl amide gel electrophoresis (PAGE) was performed as described (Laemmli, 1970). Expression of protein was quantified by scanning and computer analysis using Alphalmage200 (Alpha Innotech Corp.).

9. *Endothelial Proliferation Assay*

Different concentrations of recombinant kringles were added into the culture of bovine capillary endothelial (BCE) cells supplied with ng/ml of bFGF. The cells were maintained in DMEM medium for five days. Cells passing through an assay filter were counted.

Results

The PCR products analyzed by 2% agarose gel and EtBr-staining revealed that only a single PCR product in each reaction, indicating specific amplification performed by the hot start PCR process. The estimated PCR products encoding kringle 1, kringle 2, and kringle 1 + 2 are 304 bp. 286 bp and 550 bp, respectively. The sizes of each PCR product agreed well with those expected from tPA DNA sequence. All three PCR products were cloned into a T_7-driven vector pET11a (5.67 kb), and generated recombinant plasmids of $pK1_{tPA}$ (5.95 kb), $pK2_{tPA}$ (5.93 kb) and $pK12_{tPA}$ (6.20 kb) with a selection of ampicillin resistance. DNA sequencing indicated all constructs are in frame with the ATG start codon in the right orientation. No mutants such as substitutions, insertions or deletions occur in the tPA kringles during PCR amplification and cloning.

The tPA kringles were cloned and inserted into pET11a vector for protein expression under the control of a strong T_7 phage promoter and the laclq repressor. Recombinant plasmid $pK1_{tPA}$ encodes r-[$K1_{tPA}$], where r stands for recombinant and [KI_{tPA}] represents amino acid sequence residues [Ile86-Asn177] of kringle 1 domain in the tPA molecule. The recombinant plasmid $pK2_{tPA}$ encodes r-[$K2_{tPA}$] representing amino acid residues [Asnl77-

Thr263] of the tPA kringle 2 domain. Recombinant plasmid $pKI2_{tPA}$ encodes a r-[$KI2_{tPA}$] protein containing kringle 1 and kringle 2 domain, an internal fragment of [Ile86-Thr263] in the tPA protein sequence.

Recombinant proteins expressed in *E.coli*, were analyzed by 16% polyacrylamide gel. Whole cellular proteins of 50 μg were denatured by SDS and β-mercaptoethanol and loaded gel for electrophoresis. Abundant expression of a 10 kDa protein was detected from the cells carrying $pK2_{tPA}$ construct. 10 colonies of $pK2_{tPA}$ construct were chosen, and all ten expressed the same 10 kDa protein. A 20 kDa protein was found from the cells harboring $pK12_{tPA}$ plasmid. All ten clones of $pK12_{tPA}$ expressed the 20 kDa protein. These two proteins were produced at very high levels and occupied as high as 39.9% of the total cellular protein as indicated by a density analysis of Coomassie blue-stained PAGE gel. However, no expression was observed from the cells of ten clones containing plasmid $pK1_{tPA}$. Non expression of kringle 1 domain was confirmed by an additional screening of 20 pKI_{tPA} clones.

The DNA deduced amino acids for r-[$K2_{tPA}$] protein is a peptide of 88 residues including the initial methionine. Molecular weight (MW) of r-[$K2_{tPA}$] with an estimated weight 9.716 kDa, agrees with the size of 10 kDa analyzed from the polyacrylamide gel (Fig. 7). r-[$K12_{tPA}$] contains 179 residues with a MW of 19.810 kDa, as expected from its DNA sequence. Expression of a 20 kDa protein from the $pK12_{tPA}$ plasmid is congruent with that expected. However, even r-[$K1_{tPA}$] sharing 92 identical amino acids with the N-terminus of r-[$K12_{tPA}$], was not expressed.

High-level expression of proteins in *E. coli* frequently results in inclusion bodies, the unfolded insoluble protein particles in the cytosol of cells. Expression of human tPA kringles accumulates as inclusion bodies, as happens with most of the mammalian proteins (Dicou, 1992). These unfolded

proteins can be isolated by centrifugation. Unfolded proteins need to be solubilized, refolded and purified for testing of anti-angiogenesis activity.

Both recombinant proteins r-[$K2_{tPA}$] and r-[$K12_{tPA}$] contain tPA kringle 2 domains. The tPA kringle 2 structure has the lysine-binding site. Lysine-bind site in the tPA kringle 2, binding resins coupled with lysine ligand, can be used for affinity protein purification. The stable epoxy-activated lysine-agarose gel was used to purify the recombinant proteins r-[$K2_{tPA}$] and r-[$K12_{tPA}$]. L-arginine is a competitor of lysine ligand. Efficient binding of recombinant tPA kringles with lysine ligand was found when the concentration of L-arginine in the protein solution is reduced below 25 mM. Elution of recombinant tPA kringles was carried out using a competitive ligand of lysine, the 6-amino-n-caproic acid. Three peaks are detected in the eluted fractions from a 0-200 mM gradient, one is between 30 to 45 mM, the second is between 60 to 105 mM, and a third large peak between 125 to 150 mM of 6-amino-n-caproic acid. Samples from these fractions were subjected to SDS-PAGE analysis. The r-[$K2_{tPA}$] and r-[$K12_{tPA}$] proteins eluted in the peak of 125-150 mM 6-amino-n-caproic acid, appeared homogeneous on the denatured 16% polyacryl amide gel.

Proliferation assay using bovine capillary endothelial cells indicated that both of r-[$K2_{tPA}$] and r-[$K12_{tPA}$] proteins are active for anti-angiogenesis. However, the protein r-[$K2_{tPA}$] containing tPA kringle 2 alone showed stronger activity than r-[$K2_{tPA}$] suggesting that the tPA kringle2 is an angiogenesis inhibitor (Fig 8). A similar result has been observed from plasminogen kringles that the plasminogen 2 alone is more active than a peptide containing plasminogen kringle 2 and 3.

All five plasminogen kringles have been cloned. The anti-angiogenic activity of plasminogen kringles are ranked as kringle 5>1>3>/=2>4. The most active plasminogen kringles are, kringles 5 and 1. Plasminogen kringle 4

does nor show anti-angiogenic activity. Because analysis of anti-angiogenic activity of tPA kringles do not include plasminogen kringles, tPA kringle activity was not compared with that of plasminogen kringles. Sites for mutation in tPA kringle were selected using amino acid sequences of plasminogen kringles as references, in which plasminogen kringle 5 and 1 is the positive reference and kringles 2, 3, 4 - especially kringle 4 - is the negative reference.

Ideally, positive selection or negative selection can be designed. This is performed by aligning amino acid sequence of tPA kringle 2 with the five plasminogen kringles. Next, identify the position(s) where the amino acid residue(s) is identical between plasminogen 5 and 1, but is different from plasminogen 4 and tPA kringle 2. If a position is occupied with a similar or the same amino acid residue in tPA kringle 2 and plasminogen kringle 4, this is where the residue should be mutated with top priority. Then, change the amino acid residue(s) in kringle 2 to an amino acid that is identical in plasminogen kringle 5 and 1. Finally, express the resultant mutant and test its effects on endothelial proliferation to determine if the mutation changes (increases) anti-angiogenic activity. Alternatively, negative selection can be designed by changing a residue in tPA kringle identical with both of plasminogen kringle 5 and 1 into an amino acid identical with that of plasminogen kringle 4. Here, mutations for positive selection only are performed.

The mutagenesis procedure utilizes a super-coiled, double-stranded and methylated DNA plasmid $pK2_{tPA}$ and two synthetic oligonucleotide primers containing the desired mutation. PCR, employing Pfu, a proofreading DNA polymerase, efficiently produced, nicked and unmethylated, the daughter plasmid with mutation by uptake of the oligonucleotide primers. The methylated parent $pK2_{tPA}$ plasmid was eliminated by restriction digestion using DpnI, which is an enzyme cutting the

methylated substrate DNA. The mutated daughter plasmids were recovered by transformation into high-efficiency competent *E. coli* cells. A control PCR was accompanied with each set of mutations by excluding the oligodeoxynucleotide primers. Selection of transformants using the ampicillin resistance maker encoded by the vector, indicated that at least a hundred fold of colonies were obtained from the PCR reaction mixture containing the mutation primers compared to that of PCR excluding the primers.

Dideoxynucleotide DNA sequencing of plasmids revealed that all mutations designed were successfully introduced onto the tPA kringle DNA strand. Any undesired or unexpected mutation was not observed, suggesting that high fidelity DNA extension had been performed during the PCR process using the proofreading Pfu enzyme.

All kringle mutants should be approximately 10 kDa. Expression of mutants was analyzed on 16% polyacryl amide gel electrophoresis (Fig 9). Four of them were expressed. However, Y, N and G mutants were unstable. Interestingly, the Y, N and G mutants are all located in the middle in the kringle structure, suggesting that mutation in this region can create unstable mRNA or protein structure.

tPA kringle 2 mutants were solubilized and reduced by 8 M urea and 140 mM β-mercaptoethanol, as described in Materials and Methods. The reduced mutants were fractionated by Super-fine Sephadex G-75 chromatography in the presence of 8 M urea. The reduced mutant proteins appeared to be homogenous by denatured SDS-PAGE analysis.

S, K, H, W kringle mutants and the wild-type tPA kringle protein were tested by proliferation assay using bovine endothelial cells in the presence of bFGF. The primary structure of tPA kringle 2 and the mutation sites are shown in Figure 5. Mutation on K and W showed no effects on the anti-

angiogenic activity of tPA kringle. S mutant slightly affected the activity. However, mutation on the H site generated at least 4 times higher anti-angiogenic activity than the parental wild-type kringle, indicating that the H site can be a potent active site for expression of anti-angiogenic activity of kringle structure.

Discussion

tPA kringle 2, and tPA kringle 1 plus kringle 2 was expressed. tPA kringle 1 domain was unable to be expressed. Expression of tPA kringle 1 was only achieved by the fusion of kringle 2 domain, the r-[$K2_{tPA}$] peptide. To the best of the knowledge, peptide, containing tPA kringle 1 only, has never been expressed. Expression of tPA kringle 1 has been reported as a fusion with plasminogen kringle domain (DeSerrano et al., 1992).

EXAMPLE 8

Anti-angiogenic Mechanisms of Kringles: Cloning and Expression of tPA Kringles:

The tPA kringles have been cloned and inserted into pET11a vector for protein expression under the control of a strong T7 phage promoter and the laclq repressor. Recombinant plasmid pK1tPA encodes r-[K1tPA], where, r stands for recombinant and [K1tPA] represents amino acid sequence residues [Ile86-Asn177] of kringle 1 domain in tPA molecule. The recombinant plasmid pK2tPA encodes r-[K2tPA] representing amino acid residues [Asn177-Thr263] of tPA kringle 2 domain. Recombinant plasmid pKl2tPA encodes ar-[Kl2tPA] protein containing kringle I and kringle 2 domain, an internal fragment of [Ile86-Thr263] in tPA protein sequence.

Recombinant proteins expressed in *E. coli* were analyzed by 16% polyacrylamide gel. Whole cellular proteins of 50 μg were denatured by SDS and β-mercaptoethanol and loaded gel for electrophoresis. Abundant expression of a 10 kDa protein has been detected from the cells carrying

pK2tPA construct (Fig. 7). Ten colonies of pK2tPA constructs were selected and all ten expressed the same 10 kDa protein. A 20 kDa protein was found from the cells harboring pK12tPA plasmid. All ten clones of pK12tPA expressed the 20 kDa protein. These two proteins were produced at very high level that occupied as high as 39.9% of the total cellular protein by a density analysis of Coomassie blue-stained PAGE gel. However, no expression was observed from the cells of ten clones containing plasmid pK1tPA. No expression of kringle 1 domain was confirmed by an additional screening of 20 pK1tPA clones.

The DNA deduced amino acids for r-[K2tP A] protein is a peptide of 88 residues including the initial methionine. Molecular weight (MW) of r-[K2tPA] estimated at 9,716.42 Da, agrees with the size of 10 kDa analyzed from the polyacrylamide gel. r-[K12tPA] contains 179 residues with a MW of 19810.92 Da expected from its DNA sequence. Expression of a 20 kDa protein from the pK12tPA plasmid perfectly fits with that expected. However, even r-[K1tPA] sharing 92 identical amino acids with the N-terminus of r-[K12tPA], was not expressed.

Purification:

Both recombinant proteins, r-[K2tPA] and r-[K12tPA], contain tPA kringle 2 domains. The tPA kringle 2 structure has the lysine-binding site. Lysine-bind site in the tPA kringle 2, binding resins coupled with lysine ligand, can be used for affinity protein purification. The stable epoxy-activated

Lysine-agarose gel was used to purify the recombinant proteins r-[K2tPA] and r-[K12tPA]. The proteins were refolded in the presence of 0.5 M L-arginine as 68 described (Cao et al., 1996). L-arginine is a competitor of the lysine ligand. Efficient binding of recombinant tPA kringles with lysine ligand occurs, when the concentration of L-arginine in the protein solution has

been reduced below 25 mM. Elution of recombinant tPA kringles was carried out using a competitive ligand of lysine, the 6-amino-n-caproic acid. There are three peaks detected in the eluted fractions from a 0-200 mM gradient, one is between 30 to 45 mM, one is 60 to 105 mM and a large peak between 125 to 150 mM of 6-amino-n-caproic acid. Samples from these fractions were subjected to SDS-PAGE analysis. The r-[K2tPA] and r-[K12tPA] proteins eluted in the peak of 125-150 mM 6-amino-n-caproic acid. Samples from these fractions were subjected to SDS-PAGE analysis. The r-[K2tPA] and r-[K12tPA] proteins eluted in the peak of 125-150 mM 6-amino-n-caproic acid, appeared to be homogeneous on the denaturing 16% polyacrylamide gel.

Effects on endothelial migration by mutation:

Different concentrations of recombinant tPA kringles were added into the culture of bovine capillary endothelial (BCE) cells. The cells were maintained in DMEM medium for five days. Cells passed through an assay filter were counted. Assay results indicated that both of r-[K2tPA] and r-[K12tPA] proteins actively inhibit endothelial proliferation. Protein r-[K2tPA] containing tPA kringle 2 alone showed stronger activity than r-[K2tPA], suggesting that the tPA kringle 2 can be an angiogenesis inhibitor (Fig. 8).

A Potent Anti-angiogenic Active Site in Kringle:

Identification of active site(s) for anti-angiogenesis activity of kringles would lead to an understanding of the anti-anglogenesis mechanism, which as so far eluded investigators studying the plasminogen kringle proteins. One way to address this is to perform *in vitro* site-directed mutagenesis at various sites in the kringle domain. The tPA kringle domain is a good choice for the mutation, since: 1) tPA kringle 2 contains a strong lysine-binding site that is preferred for ease isolation of kringle mutants; 2) tPA kringle 2 has been extensively studied and much is known about its structure and function.

All five plasminogen kringles have been cloned (Cao et al., 1996). The sequence of anti-angiogenic activity of plasminogen kringles is k5 > k1 > k3 >/= k2 > k4(Cao et al., 1996; Nelson et al., 1995). The most active plasminogen kringles are kringle 5 and I. Plasminogen kringle 4 did not show activity. Because performing the analysis of anti-angiogenic activity of tPA kringles did not include plasminogen kringles, the tPA kringle activity cannot be compared with that of plasminogen kringles. Sites for mutation in tPA kringle were chosen using amino acid sequences of plasminogen kringles as references, in which plasminogen kringle 5 and 1 were selected for the positive and kringle 2, 3, 4, especially kringle 4, were selected for the negative reference.

Ideally, positive selection or negative selection can be designed. First, align the amino acid sequence of tPA kringle 2 with the five plasminogen kringles. Next, identify the position(s) where the amino acid residue(s) is identical between plasminogen 5 and 1, but is different from plasminogen 4 and tPA kringle 2. If a position is occupied with a similar or the same amino acid residue in tPA kringle 2, and plasminogen kringle 4, where the residue should be mutated is the top priority. Then, change the amino acid residue(s) in kringle 2 to an amino acid that is identical in plasminogen kringle 5 and 1. Finally, the resultant mutant is expressed and its effects tested on endothelial proliferation to see if the mutation changes (increases) its anti- angiogenic activity. Alternatively, negative selection can be designed by changing a residue in the tPA kringle identical with both of plasminogen kringle 5 and 1 to an amino acid identical with that of plasminogen kringle 4. Here in the present studies, mutations for positive selection were only performed.

The mutagenesis procedure utilizes a super-coiled, double-stranded and methylated DNA plasmid pK2tPA and two synthetic oligonucleotide primers containing the desired mutation. PCR employing Pfu, a proofreading

DNA polymerase, efficiently produced, nicked and unmethylated daughter plasmid with a mutation by uptake of the oligonucleotide primers. The methylated parent pK2tPA plasmid was eliminated by restriction digestion using DpnI which is an enzyme cutting the methylated substrate DNA. The mutated daughter plasmids were recovered by transformation into high-efficient competent *E. coli* cells. A control PCR was accompanied with each set of mutation by excluding the oligodeoxynucleotide primers. Selection of transformants using the ampicillin resistance maker encoded by the vector indicated that at least a hundred fold of colonies was obtained from the PCR reaction mixture containing the mutation primers compared to that of PCR excluding the primers.

Dideoxynucleotide DNA sequencing of plasmids revealed that all designed mutations were successfully introduced on the tPA kringle 2 cDNA. Any undesired or unexpected mutation was not observed, suggesting that high fidelity DNA extension has been performed during PCR process using the proofreading Pfu enzyme.

All kringle mutants should be approximately 10 kDa MW. Expression of mutants were analyzed on 16% polyacrylamide gel electrophoresis (Fig. 9). S (Ser186 substituted by Lys) and H (His244 substituted by Tyr) mutants were expressed. However, the Y (Tyr214 substituted by Phe) mutant was not expressed. The tPA kringle 2 protein mutants were solubilized and reduced with 8M urea and 140mM β-mercaptoethanol. Proteins were fractionated by Super-fine Sephadex G-75 chromatography in the presence of 8M urea. The reduced mutant proteins appeared to be homogenous by denatured 16% SDS-PAGE analysis. The pure reduced mutants were refolded and purified by lysine-agarose chromatography.

Effects on endothelial proliferation by mutation:

Kringle mutants S, Y, H and wild-type tPA kringle 2 proteins were

tested by proliferation assay using bovine endothelial cells. The selected mutation sites in tPA kringle 2 domain are indicated (Fig. 10). Mutation on S showed no marked effects on activity. Surprisingly, H mutant with a single amino acid substitution of histidine by tyrosine dramatically increased inhibitory activity in the endothelial proliferation assay (Fig. 19). H mutant completely inhibits endothelial cell proliferation at 40 nM. The parental wild-type tPA kringle 2 shows no activity at the same concentration. The wild-type tPA kringle inhibits endothelial proliferation only approximately 45% at 200 nM. These results indicate that the H site can be a potent active site for expression of anti-angiogenic activity of kringle structure.

Effects on corneal angiogenesis by mutation:

As can be seen in Fig. 19 the addition of 5ng of H mutant of tPA K2 to a pellet containing 25ng bFGF is capable of significantly suppressing corneal angiogenesis.

The H site might be on the surface of protein:

Residues of Asn248- Trp253 form a lysine-binding site in tPA kringle 2 domain(Bakker et al., 1993). These residues should be exposed on the surface of protein for exhibiting lysine binding of tPA kringle 2. The H site (His244) is closed to Asn248-Trp253 lysine binding site. His244 might be also exposed on the surface of kringle 2 domain for expressing anti-angiogenic activity.

EXAMPLE 9

Design of peptide overlapping anti-angiogenic active site(s) of tPA kringle.

Peptides as short as 10 amino acids of murine epidermal growth factor (EGF) have been shown to be able to inhibit endothelial cell proliferation(Nelson et al., 1995). There are two lines of evidence which strongly support the hypothesis that synthesized peptides will inhibit endothelial cell proliferation. First, peptide fragments of fibronectin, EGF and

thrombospondin have been shown to inhibit endothelial cell growth (Homandberg et al., 1985; Tolsma et al., 1993). Second, denatured human plasminogen kringle 5 even shown stronger inhibition of endothelial cell proliferation than the refolded form (Ji et al., 1998). In other words, the denatured kringle 5 can be seen as acting as a large extended peptide. Different kringles with varied anti-angiogenic activity can exist in the human genome, however, there appears to be only one human endostatin. Human endostatin and mouse endostatin are highly conserved (Standker et al., 1997).

Anti-angiogenic peptide: Design of anti-angiogenic peptide:

Peptides have been designed to overlap the H mutation site. One is the wild-type sequence. Another one is a mutant peptide with substitution of histidine by tyrosine.

Molecular Cloning & Protein Synthesis:

The KED gene was constructed by fusion of cDNA plasminogen kringle 5 encoding Glu478-Ala562 with a collagen α1 (XVIII) cDNA fragment encoding His 1154-Lys1336. The KED gene is expressed in E. coli cells. AGA codon is a rare codon used in E.coli (Wada et al., 1990). The tRNAAGA is encoded by dna Y gene. The dna Y gene also has been reported to be essential for DNA replication 72. To improve productivity of KED protein in E. coli, a vector pEXdna Y was constructed containing a strong T7 promoter for high-speed transcription of KED mRNA and a dna Y gene supplying tRNA AGA for efficient translation of the KED product. (Fig. 1) The engineered KED gene encodes 271 amino acids. Expression of KED protein from pEXdnaY-hKED, was induced by IPTG (isopropyl β-D-thiogalactopyranoside). Abundant KED production was achieved. KED protein accumulated to 47% of total cellular protein by 2 hours induction with 0.5 mM IPTG (Fig. 2). Optimal production of KED protein was performed using a fermentor. The KED protein was produced as inclusion bodies in

cytosol. KED inclusion bodies were isolated and efficiently refolded in the presence of 0.5 M L-arginine at pH 8.6. As a control, human endostatin was simultaneously expressed from pEXdna Y-hED. More than 99% of human endostatin protein precipitated in the refolding solution as has been previously observed (O'Reilly et al., 1997). No precipitation of KED fusion occurs during the refolding.

Pure KED protein was obtained by HiTrap HeparinSepharose chromatography. Analysis of KED protein by denatured SDS-PAGE (polyacrylamide gel electrophoresis) revealed a single 30 kDa peptide that agreed with the molecular weight estimated form the deduced amino acid sequence (Fig. 10). N-terminal analysis of KED protein (by Commonwealth Biotechnologies, Richmond, VA) provided an identical sequence of MEEDHMFGNGKGYRG as deduced from DNA sequence, see Sequence Listing, where the 5th residue is Cys that generally does not show up by the analysis system. Compositional analysis of KED generated similar results with that predicted, Table 1. Both of N-terminal and compositional analysis indicates that the pure protein isolated is the correct form of the KED gene product.

Endothelial Cell Proliferation:

KED - fusion Proliferation assays using bovine endothelial cells indicate that fusion protein H1 completely inhibited endothelial cells migration at a concentration as low as 8 nM (250 ng/ml), where, human endostatin shown almost no activity at 12 nM (250 ng/ml) by the same assay (Fig. 20). The IC_{50} of human endostatin in the assay was 17.5 nM (350 ng/ml). Ic50 of human plg-k5 was approximately 150 nM. Ic50 for antiostatin was 135 nM. These results indicate that the human fusion protein KED is much stronger than endostatin, the strongest anti-angiogenic agent reported to date(O'Reilly et al., 1997).

Endothelial Cell Migration: KED - fusion

To determine the ability of the test compounds to block migration of endothelial cells toward the angiogenic factor bFGF, migration assays are performed in a modified Boyden chamber using BCE cells (Dameron et al., 1994). Cells are grown in DMEM supplemented with 10% FBS and supplements at passage 15. To assess migration, the cells are starved overnight in DMEM supplemented with 0.1% HSA, harvested, D'YI D'Y2 :-lyi suspended in DMEM/BSA, plated at 106 cells/ml on the lower surface of a gelatinized membrane (Nucleopore Corp., Plesanton, CA) in an inverted Boyden chamber and incubated for 1.5 hours to allow cell attachment. The chambers are then righted, and test material added to the top well incubated for 5 hours. Membranes are then be fixed and stained and the number of cells migrating to the top o fthe filter in 10 high-powered fileds counted and bFGF at 10 ng/ml is used as a positive control.

Inhibition of Blood Vessel Growth in Rat Cornea: KED - fusion

The rat corneal assay has been perfonned to test *in vivo* efficacy of KED fusion protein. 12.5ng, of KED were applied in the presence of 25ng of bFGF. 12.5ng of KED fusion protein efficiently inhibited the growth of capillary blood vessels from the corneal bed (Fig. 5).

RESEARCH DESIGN AND METHODS

Overall Rationale for Design of Inhibitors:

The results together with the finding that the individual plasminogen kringle possesses anti-angiogenic activity, show that the triple disulphide bond kringle structure is a new family of angio-inhibitory compounds. Understanding of anti-angiogenic mechanisms of kringle structure would benefit tumor therapy. tPA kringle 2, a well characterized protein domain, is a preferred model kringle tor investigating angio-inhibitory mechanisms.

Identification of the active site(s) for exhibiting anti-angiogenic activity

of kringle would lead to understanding of anti-angiogenic mechanisms. A way to address this is to perform *in vitro* site-directed mutagenesis in tPA kringle 2 as proposed. Preliminary studies show that substitution of His244 with Tyr in tPA kringle 2 dramatically increases its anti-angiogenic activity, indicating the presence of a potentially active site involving the His244 residue. In order to identify anti-angiogenic active site(s), an additional I7 mutations in tPA kringle 2 structure were suggested.

Compared to kringle family, endostatin. a highly basic protein fragment of collagen α1(XVIII), represents another class of angio-inhibitory compounds. Kringle-like structures are not present in the endostatin molecule. Anti-angiogenic activity of endostatin can therefore be due to a different mechanism to that of kringles. Construction of fusion protein between highly active mutated tPA kringle 2 and endostatin is described. The fusion of two completely different structures of kringle and endostatin creates a powerful anti-angiogenic molecule. Preliminary studies support this hypothesis. KED, a fusion between plasminogen kringle 5 and endostatin, showed significantly higher anti-angiogenic activity than either kringle 5 or endostatin. Higher activity of this chimera can reflect expression of combined activity from kringle 5 and endostatin due to simultaneously activation of different anti-angiogenic mechanisms. There is now an H mutant of tPA kringle 2 that is much active than plasminogen kringle 5 which is the most active kringle reported (Nelson et al., 1995). Together with H mutant, performing of additional mutagenesis will not only allows the study angio-inhibitory mechanism but also provides a tPA kringle 2 mutant with optimum angio-inhibitory activity for fusion with endostatin, which, in turn, result a powerful agent for efficient tumor therapy.

Identification of Anti-angiogenic Active Site(s) of Kringle:

tPA kringle 2 is used as a model to identify the anti-angiogenic active site(s) of kringle structure. There are three reasons for the selection of tPA

kringle 2 as a model. At first, a Preliminary Study indicated that tPA kringle 2 is an anti- angiogenic active kringle. Second, substitution of a histidine residue with tyrosine (H mutant) in tPA kringle 2 surprisingly, increases anti-angiogenic activity. BEC proliferation inhibitory activity of this H mutant of tPA kringle 2 is stronger than plasminogen kringle 5 that is the most anti-angiogenic kringle reported to date. Third, kringle 2 domain, as a part of tPA and rPA, has been used in clinical therapy. Kringle 2 is an internal fragment of tPA and an N-terminal domain of tPA.

Mutation of tPA Kringle 2:

The recombinant plasmid pK2tPA containing the wild type tPA kringle 2 cDNA under aT7-driven promoter have been developed. Mutation of tPA kringle 2 is directly performed using this expression vector. No sub-cloning or other plasmid engineering is involved. A PCR-directed procedure is used which utilizes one set of complementary mutation primers overlapping the mutation-desired region. There is a recombinant plasmid pK2tPA containing the wild type tPA kringle 2 cDNA under a T7-droven promoter. Mutation of tPA kringle 2 is directly performed by using this expression vector. No sub-cloning or other plasmid engineering is involved. PCR-directed procedure is used that utilizes one set of complementary mutation primers overlapping the mutation-desired region. Mutagenesis is carried out using a mutation kit, Quick-Change (Stratagene, La Jolla, CA) with some modifications. Fifty μl PCR reaction mixture are prepared on ice. The mixture contains 20 mM Tris (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 100 ng pure super-coiled pK2tPA DNA, 200 ng each of sense and complementary mutation oligonucleotide primers, 200 μM of dNTP and 5 units of cloned Pfu DNA polymerase. A control PCR reaction will be simultaneously included in the thermal cycles by replacing both sense and complementary mutation primers with missense primers. Each PCR cycle consists of denaturation at 95°C for 0.5 minutes, annealing at 55°C for 0.5 minutes, and extension at 68°C for 18 minutes, for a total of 12 cycles.

Kringle 2 domain contains 87 amino acids that represent residues 177 to 263 in tPA. Mutations will be generated throughout the kringle 2 sequence. Mutation sites will be specifically selected based on alignments of tPA kringle 2, plasminogen kringle 4 and 5 (Fig. 5). These two plasminogen kringles serve as references guiding selection of candidate mutation sites in tPA kringle 2. Plasminogen kringle 5 is the strongest anti- angiogenic kringle (Cao et al., 1997). Kringle 4 has no anti-angiogenic activity (Urano et al., 1991). There are 24 positions, identical throughout these three kringles forming the basic kringle structure including the 3 disulphide bonds, which will not be subjected to mutation. Two groups of mutations will be performed: positive and negative selections of anti-angiogenic active site(s). A total of 17 mutants will be generated.

There are 9 positions in tPA kringle 2 occupied with the same amino acids as in plasminogen kringle 4, that will be mutated at top priority. These positions are Y181, T191 , S193, S206, D236, K240, W253, E254 and S262. These 8 amino acids will be changed to the amino acids that are identical in plasminogen kringle 5. This group of mutations is expected to generate mutants with increased activity. Mutated position(s) with increased activity should be the candidate active site(s) for expressing anti-angiogenic activity. This group of mutations represents a positive reference for mapping anti-angiogenic active site(s).

1. Change Y18I to M. Oligonucleotide primers will be
   5'-CAGTGACTGCATGTTTGGGAATGGG-3'
   and 5'-CCCATTCCCAAACATGCAGTCACTA-3'.

2. Change TI91 to K. Oligonucleotide primers will be
   5'-CCTACCGTGGCAAACACAGCCTCACC-3'
   and 5'-GGTGAGGCTGTGTTTGCCACGGTAGG-3'.
3. Change S193 to A. Oligonucleotide primers will be
   5'-CCGTGGCACGCACGCCCTCACCGAG-3'

and 5'-CTCGGTGAGGGCGTGCGTGCCACGG-3'.

4. Change S206 to A. Oligonucleotide primers will be 5'-CCCGTGGAATGCCATGATCCTGATAG-3' and 5'-CTATCAGGATCATGGCATTCCACGGG-3'.

5. Change D236 to P. Oligonucleotide primers will be 5'-GCCGGAATCCTCCGGGGGATGCC-3' and 5'-GGCATCCCCCGGAGGATTCCGGC -3'.

6. Change K240 to G. Oligonucleotide primers will be 5'-GATGGGGATGCCGGGCCCTGGTGCC-3' and 5'-GGCACCAGGGCCCGGCATCCCCATC- 3'.

7. Change W253 to Y. Oligonucleotide primers will be 5'-CGCAGGCTGACGTACGAGTACTGTG-3' and 5'-CACAGTACTCGTACGTCAGCCTGCG- 3 '.

8. Change E254 to D. Oligonucleotide primers will be 5'-GGCTGACGTGGGACTACTGTGATGTG-3' and 5'-CACATCACAGTAGTCCCACGTCAGCC- 3' .

9. Change S262 to A. Oligonucleotide primers will be 5'-GTGCCCTCCTGCGCCACCTAAGGATCC-3' and 5'-GGATCCTTAGGTGGCGCAGGAGGGCAC-3'.

There are 8 positions in tPA kringle k2 that are identical to those in plasminogen kringle 5, but which are not contained within plasminogen kringle 4. These 8 amino acids are F182, A223, W242, R249, L251, D257, V258 and P259. These 8 residues will be replaced with amino acids that are identical to those in plasminogen kringle 4. These mutations are expected to

produce less angiosuppressive activity in some cases. This group of mutations will serve as a negative reference for the identification of anti-angiogenic active site(s) of kringle domain proteins.

1. Mutate F182 to H. Oligonucleotide primers will be
5'-GACTGCTACCACGGGAATGGGTCAG-3' and
5'-CTGACCCATTCCCGTGGTAGCAGTC-3'.

2. Mutate A223 to N. Oligonucleotide primers will be
5'-CCCAGTGCCCAGAACCTGGGCCTGG-3' and
5'-CCAGGCCCAGGTTCTGGGCACTGGG-3'.

3. Mutate W242 to T. Oligonucleotide primers will be
5'-GATGCCAAGCCCACCTGCCACGTGCTG-3' and
5'-CAGCACGTGGCAGGTGGGCTTGGCATC-3'.

4. Mutate R249 to P. Oligonucleotide primers will be
5'-GTGCTGAAGAACCCCAGGCTGACGTG-3' and
5'-CACGTCAGCCTGGGGTTCTTCAGCAC -3'
.

5. Mutate L251 to V. Oligonucleotide primers will be
5'-GAACCGCAGGGTGACGTGGGAGTAC-3' and
5'-GTACTCCCACGTCACCCTGCGGTTC-3'.

6. Mutate D257 to N. Oligonucleotide primers will be
5'-GTGGGAGTACTGTAACGTGCCCTCC-3' and
5'-GGAGGGCACGTTACAGTACTCCCAC- 3'.

7. Mutate V258 to L. Oligonucleotide primers will be
5'-GAGTACTGTGATCTGCCCTCCTGCTC-3' and
5'-GAGCAGGAGGGCAGATCACAGTACTC- 3'.

8. Mutate P259 to K. Oligonucleotide primers will be 5'-GTACTGTGATGTGAAGTCCTGCTCC-3' and 5'-GGAGCAGGACTTCACATCACAGTAC-3'.

These 17 tPA kringle2 cDNA mutants will be sequenced before protein expression.

Expression of tPA Kringle 2 mutants:

Recombinant plasmids of 17 mutants will be transformed into expression strain E.coli bL2I (DE3) cells by the standard CaCl2 procedure. E..coli BL21 (DE3) cells will be co-transformed with a low-copy plasmId pDC952, a derivative of pACYC 184 containing argU gene. Ten colonies with ampicillin and chloramphenicol resistance for each construct will be selected for gene expression analysis. Each single colony will be inoculated in 2 ml of LB medium containing 100 ug/ml ampicillin and 35ug/ml chloramphenicol. After overnight incubation at 37°C, 0.1 ml culture from each sample will be diluted into pre-warmed (37°C) 10 ml LB, and continued growth will be facilitated with shaking of 250 rpm. Protein expression will be induced at the early log phase between 0.4 to 0.6 OD at 600 nm. by adding of IPTG (isopropyl β-D-thiogalactopyranoside) to the final concentration of 0.5 nM. After 2 hours induction, the cultures will be chilled on ice and centrifuged at 4°C. The resulting cell pellets will be washed once by PBS (phosphate saline buffer) and subjected to 20% SDS-PAGE analysis. Large-scale expression of protein will be carried out using a fermentor (Bioflo2000, New Brunswick Scientific, New Brunswick. NJ). The injection of air to the fermentor will be maintained at 15 1 air/1 culture/min. The medium used is LB enriched with 35 mg/ml yeast extract. Anti-foam agent 289 (Sigma, St, Louis, MO) will be added to the medium at 0.005% (v/v) to prevent foam-formation during fermentation. Protein induction will be performed by 1 mM IPTG at 0.5 OD (600 nm). Cells will be collected by a filtration using 0.04 μm polyethersulfone

membrane (North Carolina SRT, Cary, NC).

Purification of tPA Kringle 2 Mutants:

Expression of wild-type or H mutant of kringle 2 resulted in inclusion bodies in the Preliminary Study. These 17 mutants of kringle 2 can also form the inclusion bodies. These inclusion bodies will be dissolved in 100mM Tris (pH 8.6) containing 8M urea, 1 mM EDTA, 0.1 % Tween-20 detergent, 145mM of β-mercaptoethanol at the concentration of 10 mg/ml of proteins. These mutant proteins will be isolated by gel-filtration chromatography in the denatured condition, which was introduced for purification of wild-type tPA kringle 2 protein. Super-fine Sephadex G- 75 (Pharmacia, Piscataway, NJ) of 10g will be equilibrated in 100mM Tris (pH 8.6) containing 1mM EDTA and 8M urea at 37°C for 24 hours . The resins are packed on a columns (250nm x l5nm) at 28-30°C for preventing precipitation of urea. The reduced tPA kringle 2 mutants will be added into refolding solution containing 100 mM Tris (pH 8.8), 1 mM EDTA, 0.1% Tween-20. 0..5 M L-arginine, 1mM of reduced gluthionine (GSH) and 0.1mM oxidized gluthionine (GSSG). The protein concentration in the refolding solution will be adjusted to 100 mg/l. The refolding reaction will require 24 hours at 23°C.

The refolded protein solution will be concentrated by using ultra-filtration system consisting ofTRIPORT Module/316L Stainless Plates with 0.1 m^2 working surface and a regenerated cellulose membrane of 5 kDa MWCO (molecular weight cut-off) (North Carolina SRT, Cary, NC). The concentrated protein solution will be diluted 20 times into pre-cold(4°C) 100mM Tris (pH 8.0) solution. The final concentration of L-arginine should be 25mM that does not effect protein binding to the lysine ligands. The diluted protein solution will be then applied on a lysine-agarose column pre-equilibrated with the same 100 mM Tris at pH 8.0. Approximately, 1ml of lysine-agarose gel will be used for absorption of lmg of kringle 2 mutants. The tPA kringle 2 mutants will be eluted from the column by using a gradient

from zero to 200mM of strong lysine competitor, 6-amino-n-caproic acid. Proteins eluted from lysine column will be monitored at 280nm and analyzed by 20% SDS-PAGE. E.coli cells secrete endotoxin. Prevention of endotoxin contamination will be ensured by using Acticlean Etox (Sterogene, Arcadia, CA) chromatography. The pure recombinant tPA kringle 2 mutants will be dialyzed against 1000 fold of PBS (v/v) and loaded on an Acticlean Etox column previously washed with 1 M NaOH and equilibrated with PBS solution. One ml of Acticlean-Etox gel will be used for every l0mg of tPA kringle mutants. Finally, kringle 2 mutants will be sterilized by a 0.2 μm filter. There are total of 19 proteins, including wild-type kringle 2 and H mutants, which will be purified.

Mapping of Anti-angiogenic Active Site(s):

There are 18 mutants (including H mutant) throughout the tPA kringle 2 sequence. Anti-angiogenic activity of these mutants will be secreted *in vitro* by endothelial cell proliferation assay. Hot spot(s)' are identified for expression anti-angiogenic activity. Mutations occurring at the 'hot spot(s)' will affect the activity. Increased or decreased activity will be quantitated by endothelial cell proliferation assays, using different concentrations of kringle 2 mutants. Results will reveal the position of 'hot spot(s)' exhibiting enhanced or reduced anti-angiogenic activity, functionally isolating key structural sites.

To localize structural element(s) and/or animal sequence(s) in human tissue plasminogen (tPA) kringle domain 2 which inhibit endothelial cell proliferation and/or migration, and corneal angiogenesis. Polypeptide fragments of tPA kringle 2 containing functionally active anti-angiogenic site(s) exhibit anti-angiogenic activity. Peptides containing the putative anti-angiogenic active site(s) are synthesized. The size for the peptides ranges from 10 to 15 amino acids. Peptides as short as 10 amino acids of murine epidermal growth factor (EGF) have been shown to inhibit endothelial cell proliferation.

The amino acid sequence of tPAkringle 2 ($K2_{tPA}$; 86 residues), shown below, has certain unique chemical and enzymatic cleavage sites which are exploited to generate polypeptide fragments for functional tests. The fragmentation of $K2t_{PA}$ into polypeptides is carried out under two different conditions. First, the intact functionally active protein is subjected to limited cleavage under mild conditions without any prior modifications to the protein structure. Thus, the chances of preserving and localizing any structural elements which could be present in the native state compact regions, usually joined by more accessible or flexible regions. is increased. Secondly, the fragments to be single linear polypeptides and therefore reduce all disulfide bridges and carboxymethylate the CysSH groups of the protein before performing the cleavage reaction. More than ten polypeptide fragments covering different regions of $K2_{tPA}$ can be obtained by a combination of chemical and enzymatic cleavage methods.

Amino Acid presentation of tPA kringle 2,($K2_{tPA}$)

NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCST

Amino Acid presentation of tPA kringle 2.($K2_{tPA}$)

NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCST

Residues which are identical in tPA (two kringles) and plasminogen (five kringles) kringle domains are indicated by asterisks; the six cysteines involved in the three disulfide bridges of $K2_{tPA}$ are in bold letters.

The single methionyl bond in $K2_{tPA}$ is cleaved with excess cyanogen bromide (CNBr) in 0.1*N* HCl or 70% formic acid to produce two peptide fragments. The volatile CNBr, dilute acidic medium *(0.1NHCl* or formic acid) and the only by-product, methyl thiocyanate, are all easily removed by lyophilization. Under appropriate conditions most proteins are cleaved in yields exceeding 80%, and often quantitatively.

Cyanogen Bromide,. cleavage after M (Met)

NSDCYFGNGSAYRGTHSLTESGASCLPWNSM
ILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCST

Enzymatic fragmentation of $K2_{tPA}$ at its two glutamyl bonds are performed with the GluV8 form of glutamyl endopeptidase 1, at optimal conditions to yield three peptides. The reaction is stopped with Cbz-Leu-Leu-Glu-CH_2Cl and the fragments separated by hplc.

Glutamyl endopeptidase -!Staph. aureus V-8 Protease); cleavage after E(Glu)

NSDCYFGNGSAYRGTHSLTE
SGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWE
YCDVPSCST

Prolyl oligopeptidase is used to cleave specifically the Pro-Xaa bonds in $K2_{tPA}$. Since this protease cleaves only small polypeptides, it is used in combination with CNBr and GluV8 to generate smaller fragments. However, if $K2_{tPA}$ is susceptible to this post-proline cleaving enzyme a 27 -residue, three 16-18 residue and two small (4-6 residue) peptide fragments spanning the $K2_{tPA}$ domain can be obtained.

Post-Proline cleaving enzyme; cleavage after P. (Pro)

NSDCYFGNGSAYRGTHSLTESGASCLP
WNSMILIGKVYTAQNP
SAQALGLGKHNYCRNP
DGDAKP
WCHVLKNRRLTWEYCDVP
SCST

All peptides are purified by preparative reverse phase hplc and characterized by analytical reverse phase hplc, peptide sequencing and mass spectrometry. The kringle fragments are then tested in a series of *in vitro* (endothelial proliferation and migration) and *in vivo* (corneal angiogenesis) biological assays for their anti-angiogenic properties.

Methods: *Chemical and Enzymatic Limited Protein Digestion:* The highly specific cleavage of the methionine bond in K2 is performed by adding

a 10- fold molar excess of CNBr to the protein in 0.1 *N* HCl. Timed aliquots of the reaction mixture are taken, the volatile CNBr removed under a stream of N2 gas (in a fume hood) and the residue is analyzed by analytical hplc to determine the extent of cleavage. After the optimal conditions for the Pro-Xaa cleavage are determined, the scale of the reaction is increased, the reaction products lyophilized and purified by preparative hplc to obtain enough material for the biological assays. S. *aureus* V8 (ICN ImmunoBiochemicals, Lisle, IL), and post-proline protease digestions of $K2_{tPA}$ are carried out using 1:100 w/w protease to substrate ratio for 18 hours at 37°C in 0.1 mM phosphate buffer (V8) or $NaHCO_3$ (post-Pro), pH 8.0. The reaction is stopped by adding 100 μM Cbz-Leu-Leu-Glu- CH_2Cl (V8) or Cbz-Leu-Leu-Pro-CH_2Cl (post-Pro) for 30 min, and the reaction mixture is lyophilized. Under the reaction conditions which require unfolding of the protein, the disulfide bridges are reduced with dithiothreitol in 6.0 M guanidine solution at 50 degrees C for 30 min. The free CysSH groups are then carboxymethylated with a large excess of iodoacetic acid. Guanidine is removed by dialysis. For CNBr cleavage of the unfolded protein, the reduced protein solution in guanidine is dialyzed against formic acid. When the concentration of formic acid reaches 70 %, the modified protein is ready for CNBr cleavage. Analytical hplc-MS is performed by injecting into an Applied Biosystems aquapore C-8, 300e reversed phase column using a gradient over 30 minutes, from 0.1% TFA in water to 70% acetonitrile in water with 0.06% TFA. A flow rate of 30 El/minutesis used with a post column split diverting 2 El/min into the mass spectrometer. Spectra are accumulated with a scan rate of 400 amu/sec from 200 to 2000 with a nominal resolution of 1000. Preparative hplc to purify large quantities of protein fragments for biological assays are performed as described below in the peptide synthesis section.

Peptide synthesis: Peptides are synthesized by manual solid phase peptide synthesis using 9- Fluorenylmethyloxycarbonyl (Fmoc)*tert*-butyl chemistry as described previously. Fmoc-protected amino acids are activated

with 2-(1*H*-benzotriazole-1-yl)-, 1 ,2,2-tetramethyluronium hexafluorophosphate / 1-hydroxybenzotriazole / N;N-diisopropylethylamine (1:1:2) in 1-methyl-2-pyrrolidone / dimethylformamide / dichloromethane (1:1:1) for all coupling steps. The peptides are cleaved with reagent K (0.1 ml dimethylsulfide, 0.2 ml 1,2-ethanedithiol, 0.46 g phenol, 0.2 ml thioanisole, 0.2 ml water and 7 ml trifluoroacetic acid) for 2 hours at room temperature. Peptides are purified by reverse-phase HPLC on a Nucleosil C_8 column (30 mn, 10 μ; 250 mm x 55 mm, Macherey and Nagel) and examined for purity by analytical reverse-phase HPLC on a Nucleosil C_8 column (3 μ, 12 mn; 120 mm x 2.1 mm) as described previously (Angali et al., 1995) The samples containing the pure peptides are collected and lyophilized. Electrospray mass spectrometry is performed to confirm the analytical data. i.e. to confirm the amino acid sequences and the absence of modified products resulting from incomplete deprotections and side chain reactions with scavengers.

Analytical Protein Chemistry: Mass spectrometry, protein sequencing and amino acid analysis are performed at the Protein Structure and Carbohydrate Core Facility of the University of Michigan Multipurpose Arthritis and Musculoskeletal Diseases Center (UM-MAC), where Dr. Anagli has an appointment as a Faculty Participant and an Approved User of several Core Facilities (Center Grant Number NIH 5 P60 AR20557). Electrospray ionization mass spectra are generated using a Finnigan MAT mode 710 triple quadrupole mass spectrometer (Finnigan MAT, San Jose.. CA) with a 20 kV conversion dynode and a 4000 m/z mass range. The mass accuracy of the data acquired is routinely better than 0.01 %. Edman degradation is performed on an Applied Biosystems 470A Sequencer and a 120A phenylthiohydantoin derivative analyzer using standard cycles supplied by the manufacturer. Protein and peptide concentrations are determined precisely by quantitative amino acid analysis using an Applied Biosystems Derivatizer 420A with on-line PTC detection with the Applied Biosystems Analyzer 130A.

Throughout this application, various publications, are referenced by author and year. Full citations for the publications are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

Table 1: Compositional analysis. Amino acid residues per KED molecule predicted from DNA sequence are compared with the results from experimental analysis.

Amino Acid		Predicted (Count/mol.)	Compositional Analysis (Residues/mol.)
Ala	A	24	22.83308
Arg	R	21	21.17712
Asn	N	9	20.22813 (AsN + Asp)
Asp	D	14	20.22813 (Asn + Asp)
Cys	C	10	N/A
Gln	Q	11	20.78861 (Gln + Glu)
Glu	E	12	20.78861 (Gln + Glu)
Gly	G	25	27.36785
His	H	10	8.19698
Ile	I	7	6.66840
Leu	L	22	23.80118
Lys	K	9	7.25435
Met	M	5	4.35643
Phe	F	12	10.84014
Pro	P	18	10.55353
Ser	S	21	18.97343
Thr	T	15	13.24128
Trp	W	6	N/A
Tyr	Y	8	7.14608
Val	V	12	16.67420

Table 2

Mice #	11-17-98 (mm)	11-19-98 (mm)	11-21-98 (mm)	11-23-98 (mm)	11-25-98 (mm)	11-27-98 (mm)	11-29-98 (mm)	12-1-98 (mm)	12-3-98 (mm)	12-5-98 (mm)	12-7-98 (mm)	12-9-98 (mm)
Control PBS 0.1 ml/Day												
1	5x5 65mm³	5x6 78mm³	5x6 78mm³	6x6.7 125mm³	6x7 131mm³	7x8 204mm³	8x8.5 217mm³	8.5x9 338mm³	8.5x9.2 346mm³	9x9.5 400mm³	9.8x10 499mm³	11x11 692mm³
	6x9 169mm³	6x10 187mm³	7x11 280mm³	8x12 399mm³	8x13 433mm³	8x13 433mm³	9x13.5 569mm³	10x14.5 728mm³	10x15 780mm³	10.5x15 861mm	11xx15.5 975mm³	11x16.5 1038mm³
2												
KYH1010, 1 mil (0.1 mg/ml)/Day												
1	3x4 19mm³	4x4 33mm³	3x4 19mm³	4x5.5 46mm³	4x6 50mm³	4x6 50mm³	4x5 42mm³	4x4 33mm³	3.5x4 26mm³	3x4 19mm³	3x3.8 18mm³	3x4 19mm³
	9x10 421mm³	10x10 520mm³	11x11 692mm³	12x12 899mm³	12x13 973mm³	13x13 1142mm³	13x15 1318mm³	13.2x14 1268mm³	14x15 1529mm³	11x16 1631mm³	15.5x16.5 2061mm³	15.5x17 2124mm³
2												

Group 2 1251n (1000000 Cells / 0.1ml) injected on 10-28-1998

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CLAIMS

What is claimed is:

1. A pharmaceutical composition comprising at least one kringle protein and a pharmaceutically acceptable carrier.
2. The pharmaceutical composition according to claim 1, wherein said kringle protein is at least one from the group consisting essentially of kringle proteins 1 through 5.
3. A pharmaceutical composition comprising a molecule from the group consisting essentially of KED or a kringle derived from the tPA protein and a pharmaceutically acceptable carrier.
4. A method of treating a tumor comprising the step of administering an effective amount of at least one from the group consisting essentially of kringle proteins 1 through 5, KED and a kringle derived from the tPA protein.

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A01N 37/18, A61K 38/00, C07K 1/00, 14/00, 16/00, 17/00, 2/00, 4/00, 5/00, 7/00

A1

(11) International Publication Number: WO 00/49871

(43) International Publication Date: 31 August 2000 (31.08.00)

(21) International Application Number: PCT/US00/04798

(22) International Filing Date: 24 February 2000 (24.02.00)

(30) Priority Data:

60/121,341	24 February 1999 (24.02.99)	US
60/121,633	25 February 1999 (25.02.99)	US
60/166,176	18 November 1999 (18.11.99)	US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: AN ANTI-ANGIOGENIC KRINGLE PROTEIN AND ITS MUTANTS

(57) Abstract

There is provided a pharmaceutical composition including at least one kringle protein and a pharmaceutically acceptable carrier. Also provided by the present invention is a method of treating a disease by administering an effective amount of a compound from the group consisting of kringle proteins, KED and a kringle derived from the tPA protein.

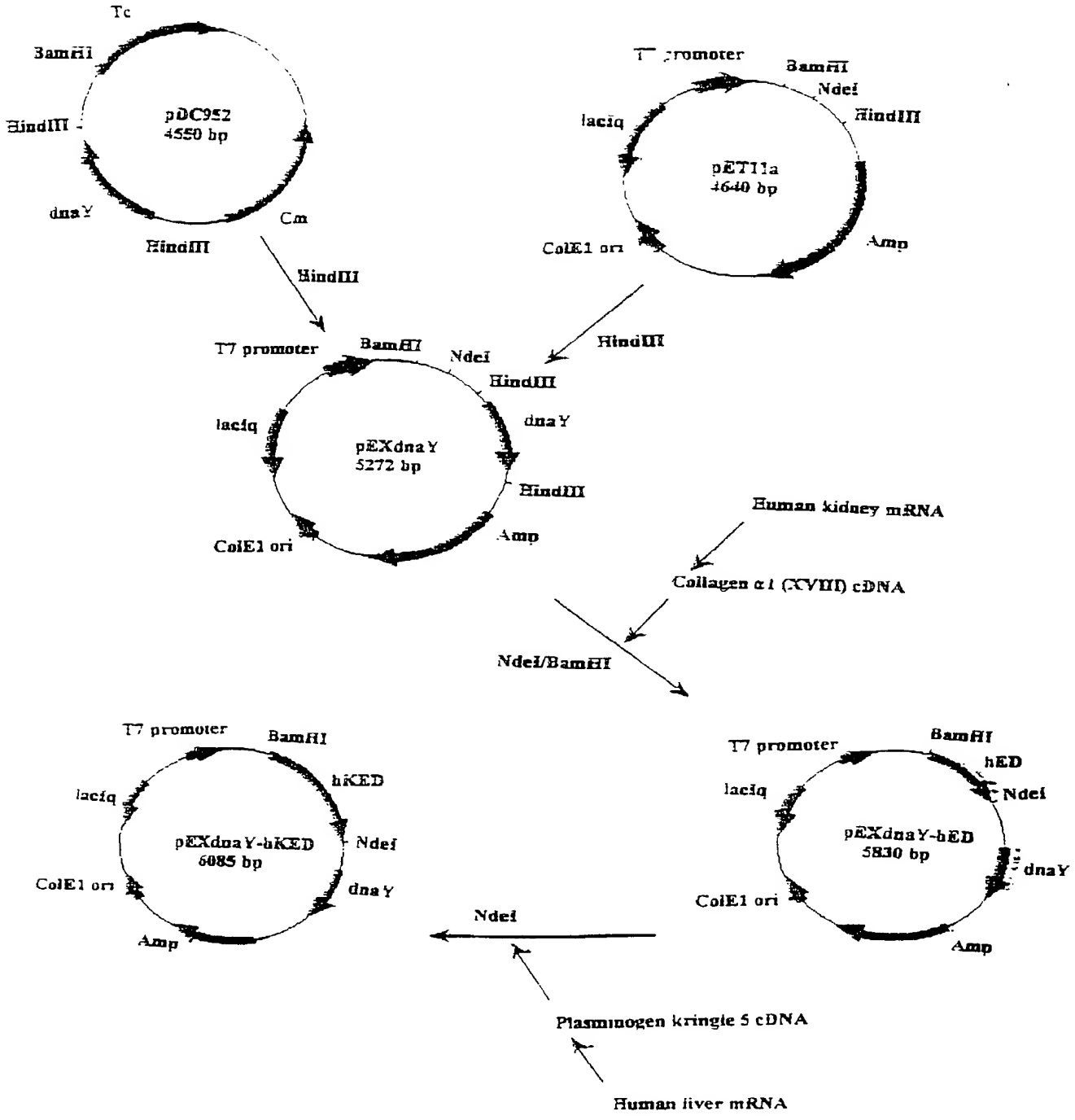

Tc
BamHI
HindIII
pDC952
4550 bp
dnaY
Cm
HindIII
HindIII
T7 promoter
BamHI
NdeI
HindIII
lacIq
pETI1a
4640 bp
ColE1 ori
Amp
HindIII
T7 promoter
BamHI
NdeI
HindIII
lacIq
pEXdnaY
5272 bp
dnaY
HindIII
ColE1 ori
Amp
Human kidney mRNA
Collagen α1 (XVIII) cDNA
NdeI/BamHI
T7 promoter
BamHI
hKED
lacIq
pEXdnaY-hKED
6085 bp
NdeI
ColE1 ori
dnaY
Amp
T7 promoter
BamHI
hED
lacIq
NdeI
pEXdnaY-hED
5830 bp
dnaY
ColE1 ori
Amp
NdeI
Plasminogen kringle 5 cDNA
Human liver mRNA

Fig. 1

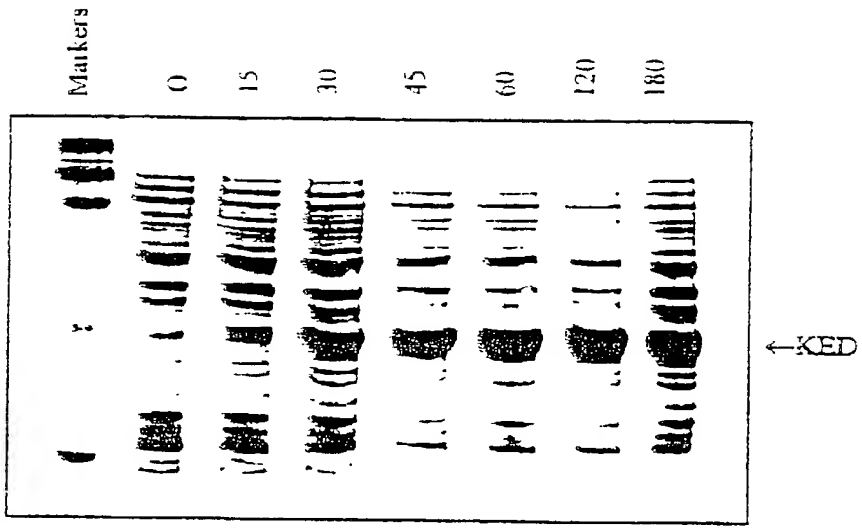

Induction Time (min)
Markers
0
15
30
45
60
120
180
←KED

Fig. 2

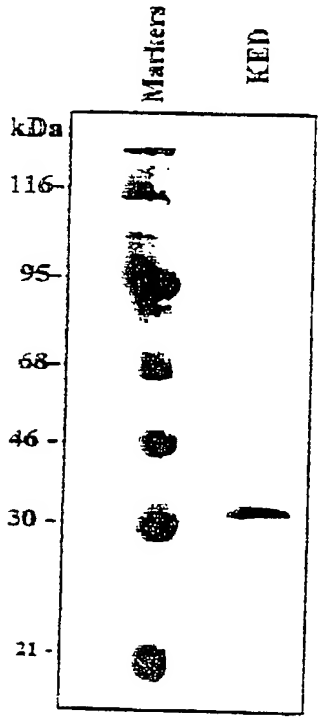
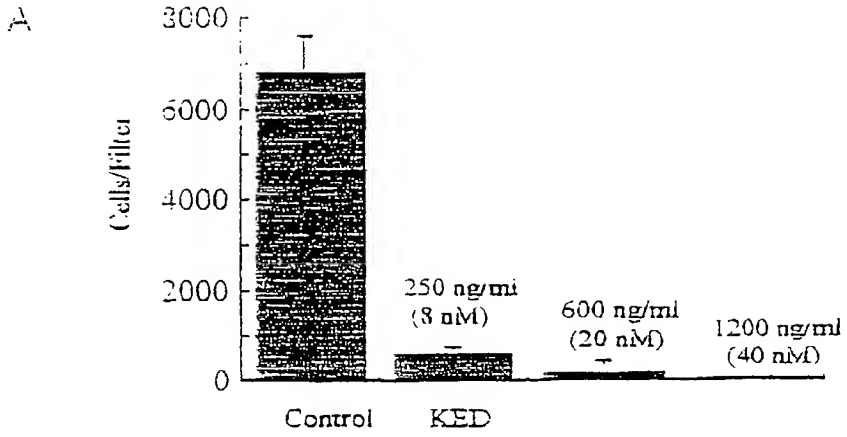

Markers
KED
kDa
116-
95-
68-
46 -
30 -
21 -

Fig. 3


A
3000
6000
4000
2000
0
Cells/Filter
250 ng/ml
(8 nM)
600 ng/ml
(20 nM)
1200 ng/ml
(40 nM)
Control
KED

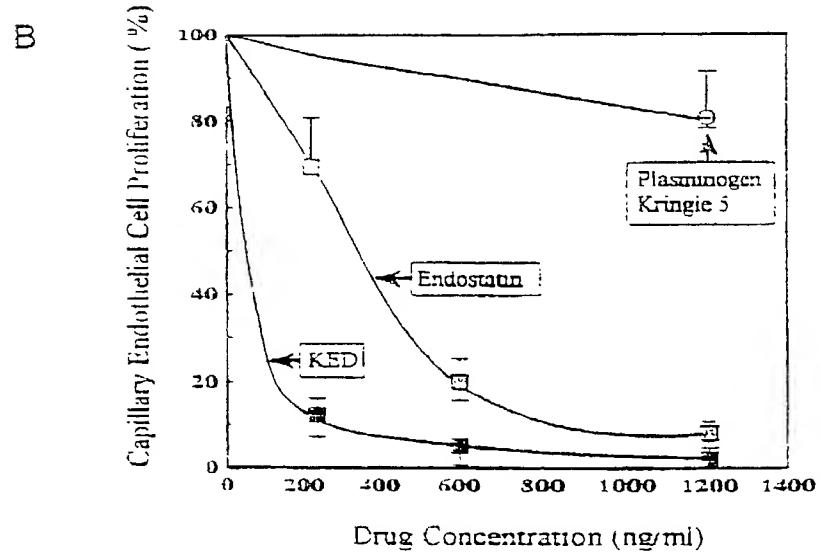

B
Capillary Endothelial Cell Proliferation (%)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Plasminogen
Kringle 5
Endostatin
KED
Drug Concentration (ng/ml)

Fig. 4

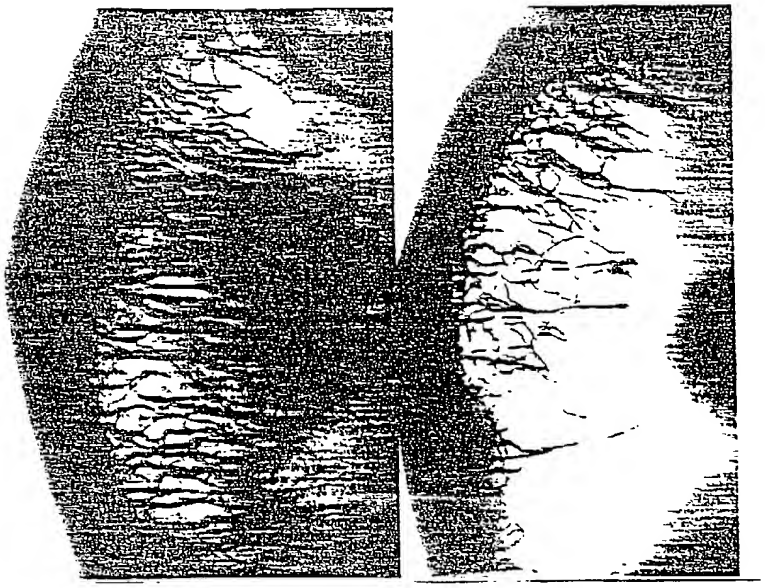


Fig. 5

Fig. 6

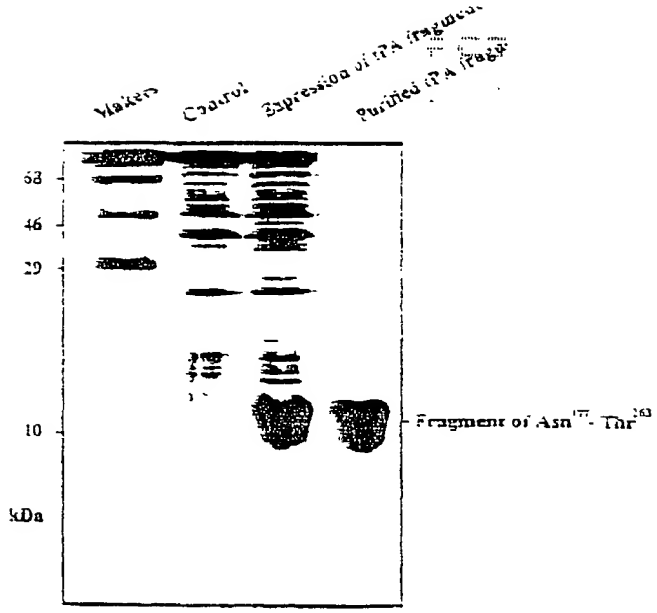

Marker
Control
Expression of tPA fragment
Purified tPA fragment
68
46
29
10
kDa
Fragment of Asn177- Thr263

Fig. 7

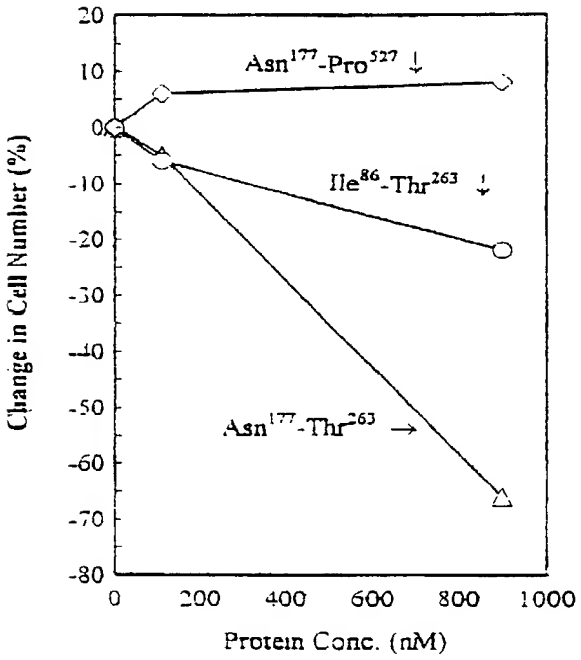

Change in Cell Number (%)
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
200
400
600
800
1000
Protein Conc. (nM)
Asn177-Pro527
Ile86-Thr263
Asn177-Thr263

Fig. 8

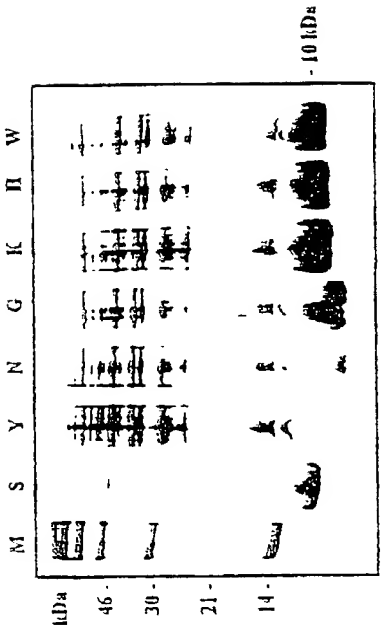


Fig. 9

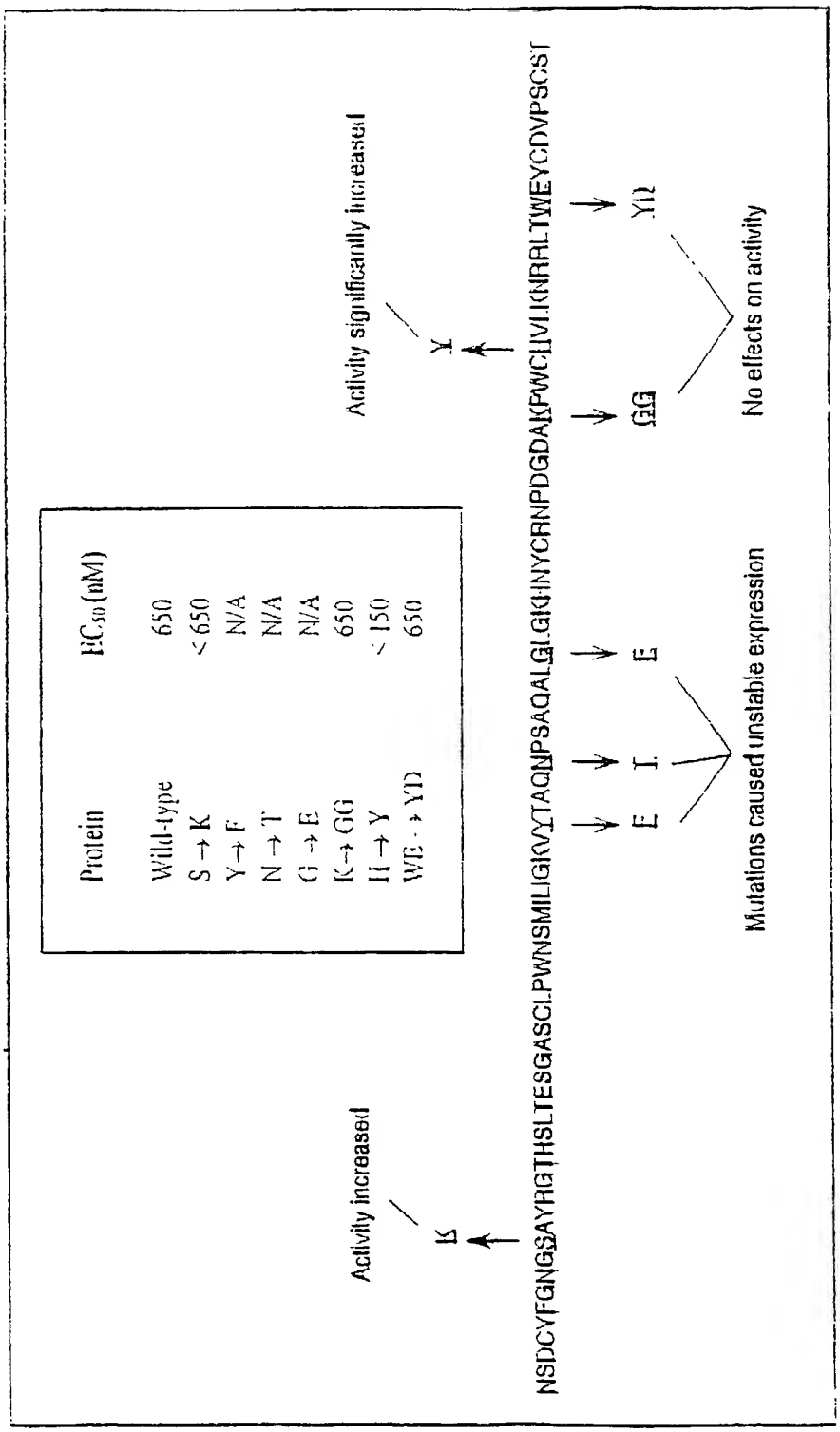

Protein
EC50 (nM)
Wild-type 650
S → K < 650
Y → F N/A
N → T N/A
G → E N/A
K→ GG 650
H → Y < 150
WE → YD 650
Activity increased
K
NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCST
Y
Activity significantly increased
E T E
Mutations caused unstable expression
GG YD
No effects on activity

Fig. 10

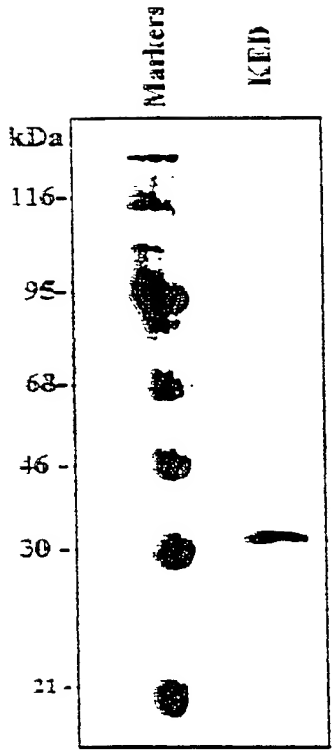
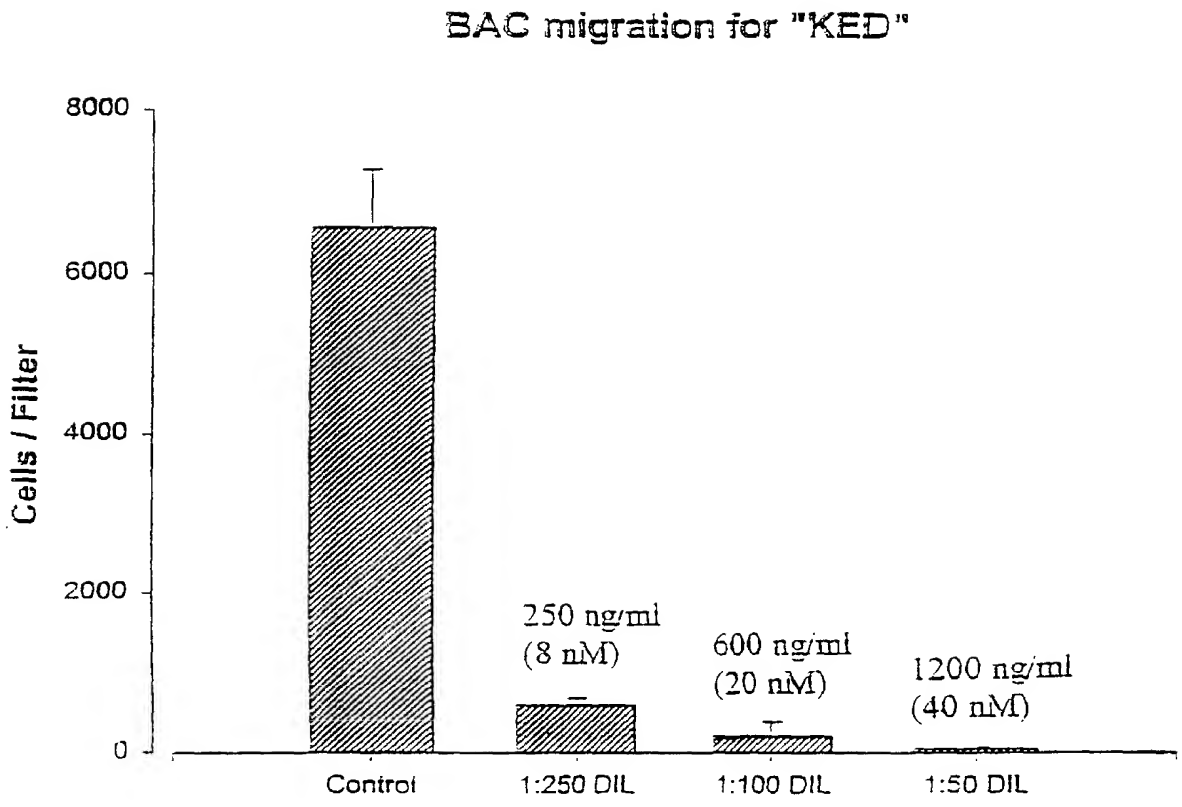
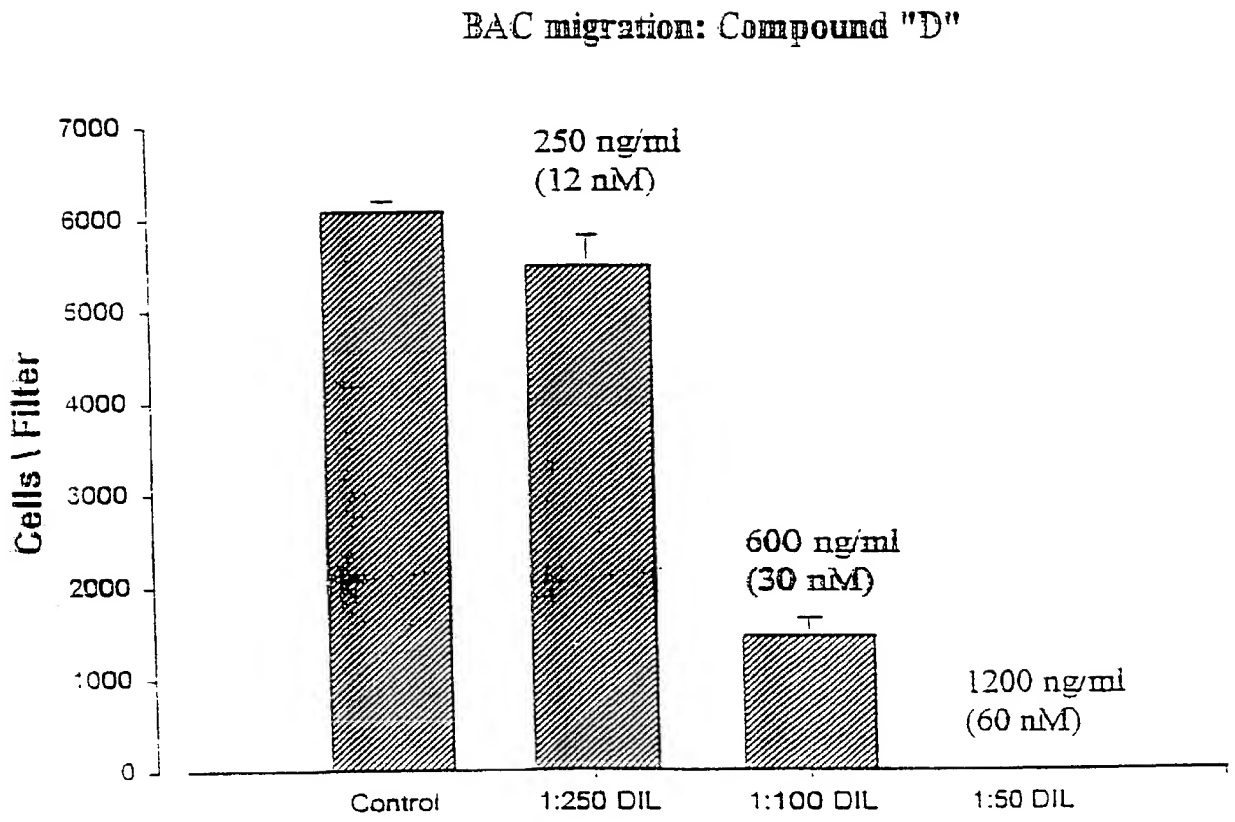


Fig. 11



MW (molecular weight) of fusion KED = 3001.25 Da

Fig. 12



MW (molecular weight) of Endostatin = 20225.87 Da

Fig. 13

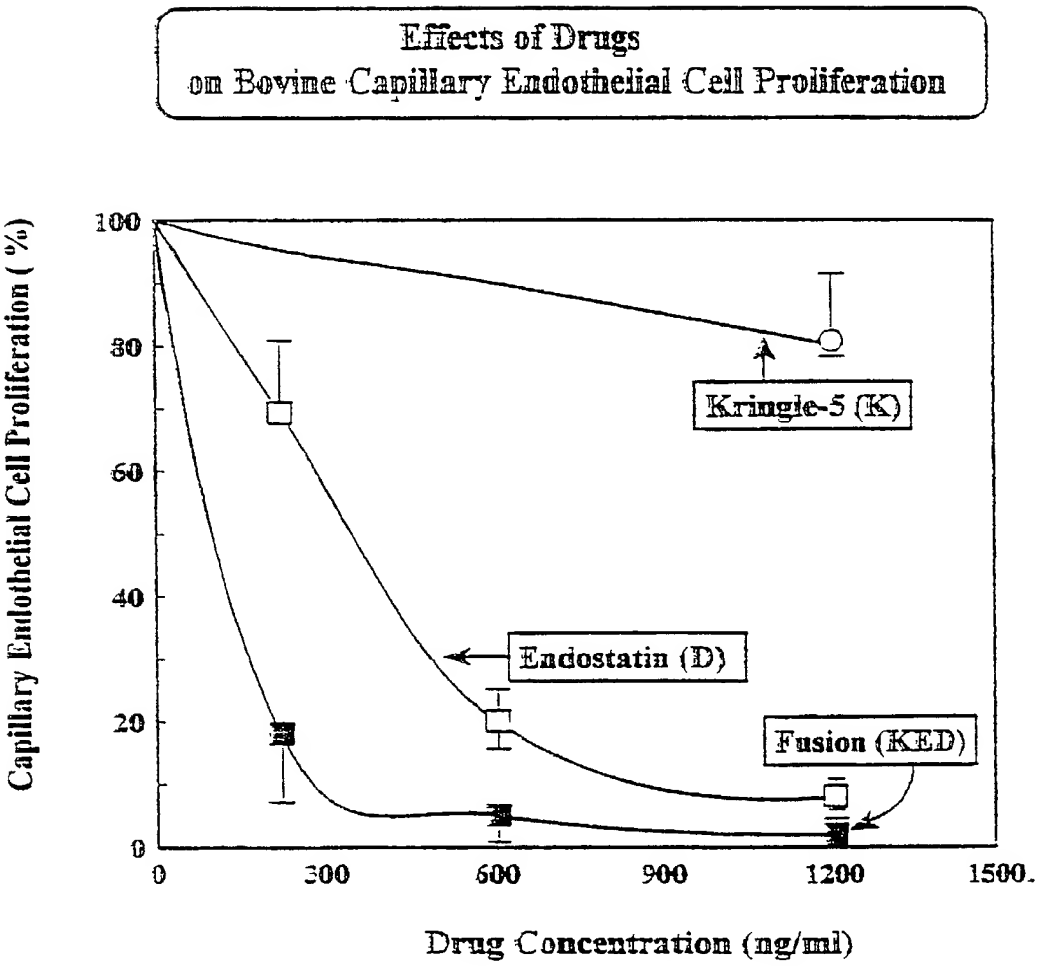

Effects of Drugs
on Bovine Capillary Endothelial Cell Proliferation
Capillary Endothelial Cell Proliferation (%)
100
80
60
40
20
0
0
300
600
900
1200
1500.
Drug Concentration (ng/ml)
Kringle-5 (K)
Endostatin (D)
Fusion (KED)

Fig. 14

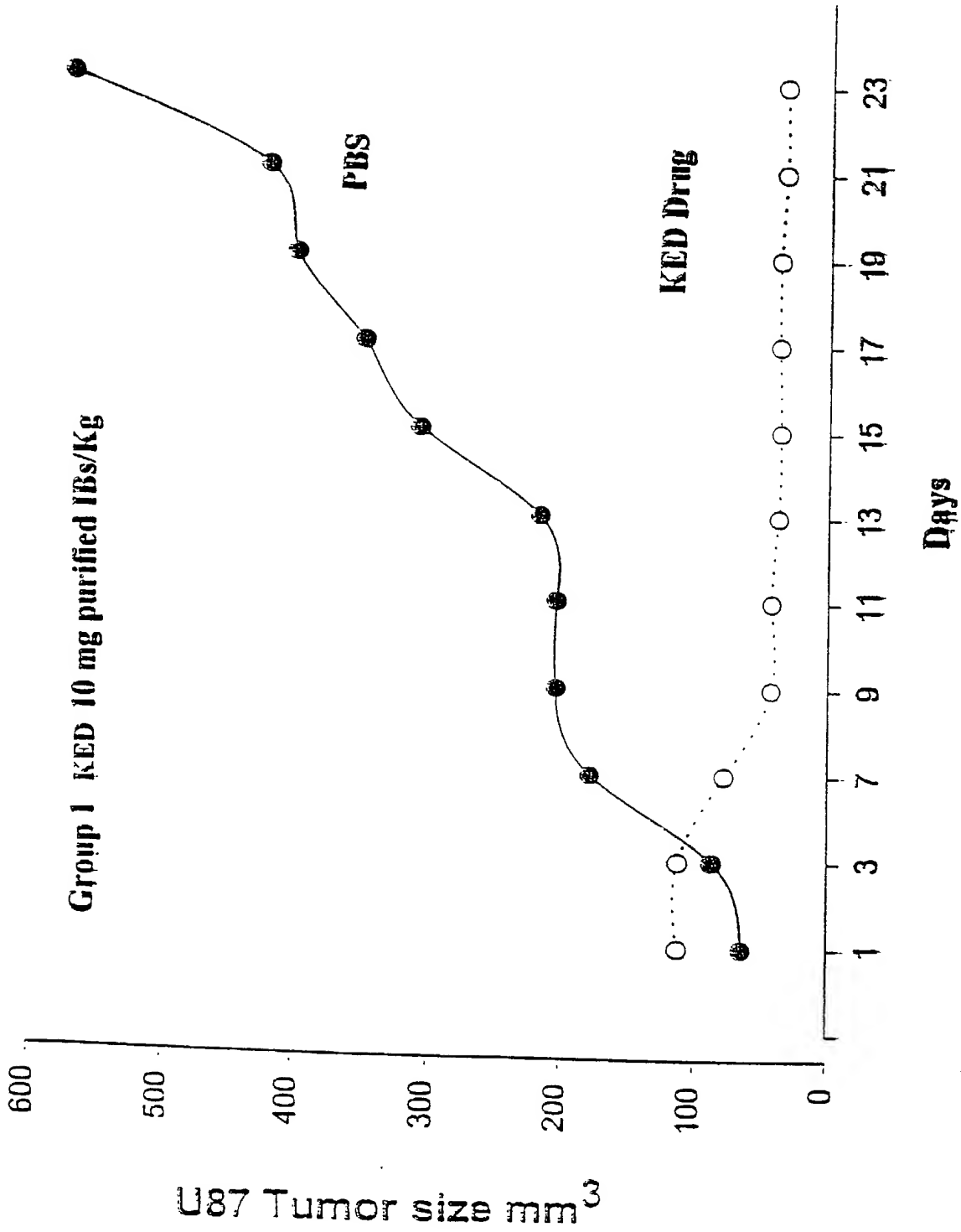

Group 1 KED 10 mg purified IBs/Kg
PBS
KED Drug
U87 Tumor size mm3
Days
600
500
400
300
200
100
0
1
3
7
9
11
13
15
17
19
21
23

Fig. 15

Group 2 KED 0.3 ml(0.1 mg/ml)/D

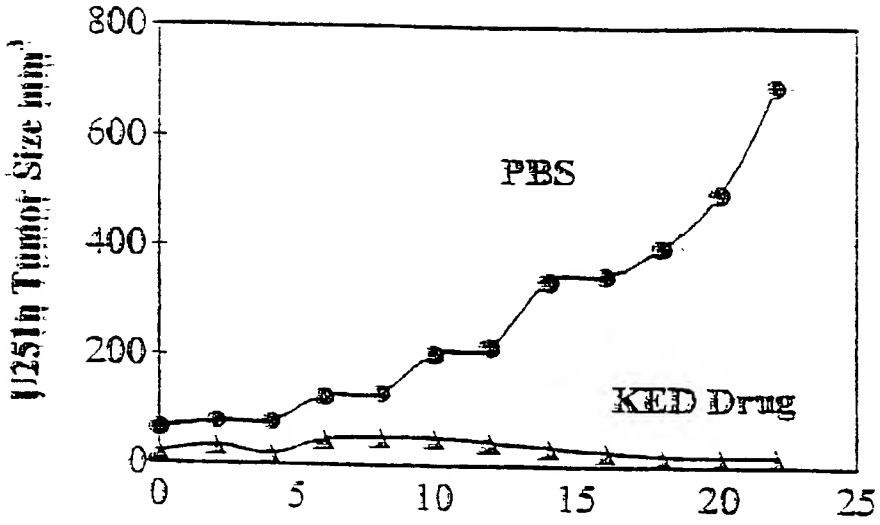


Fig. 16

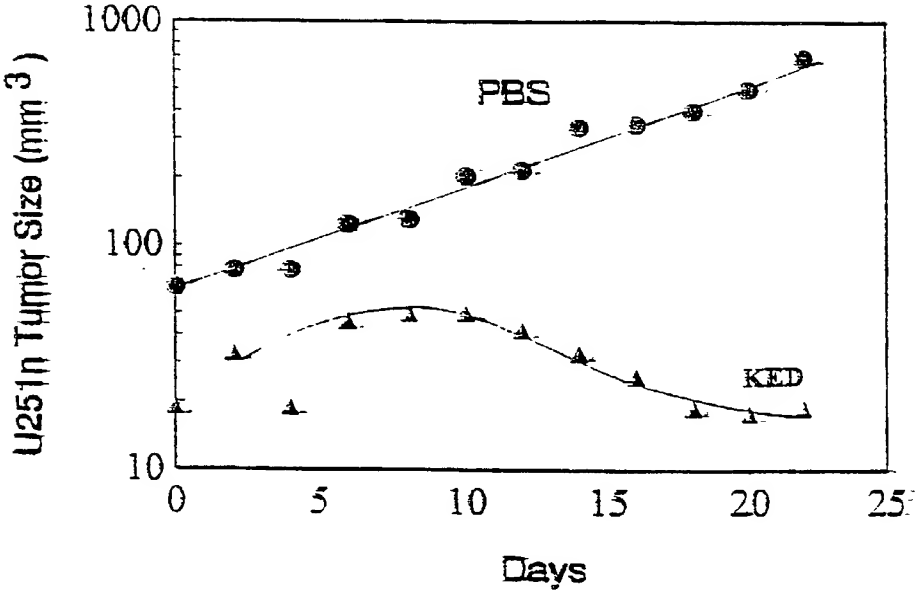


Fig. 17

Fig. 18

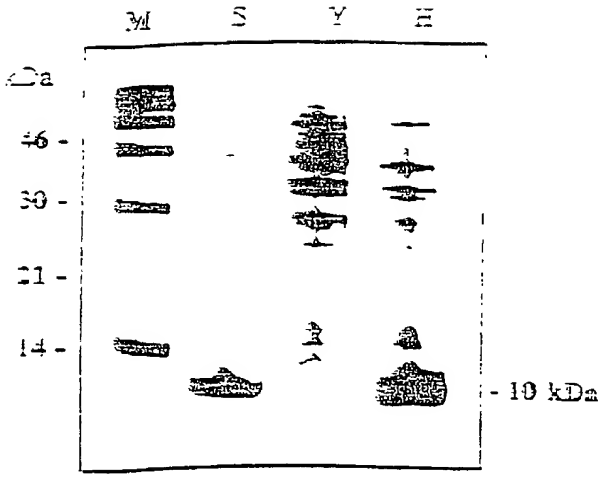
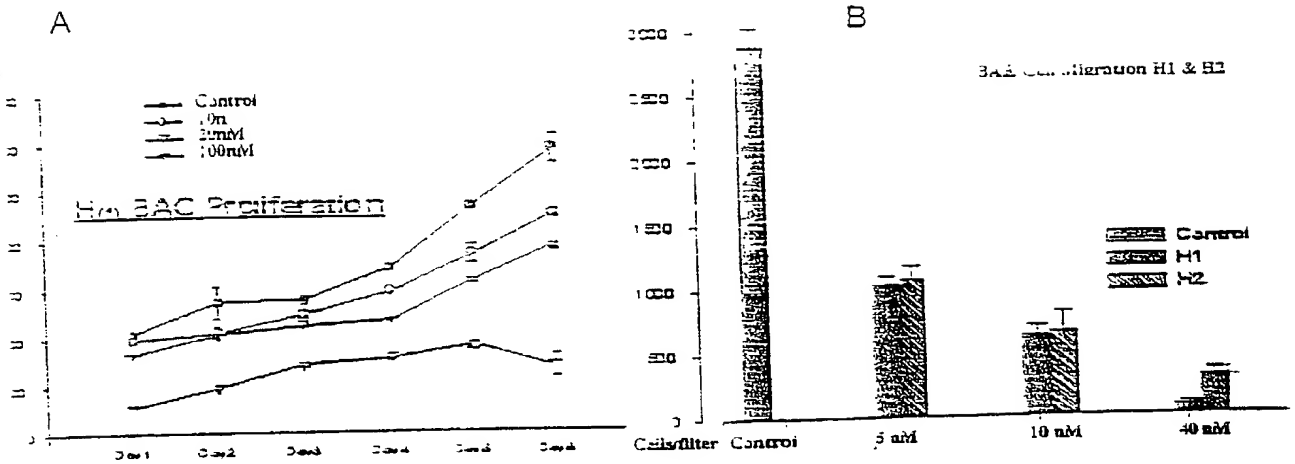
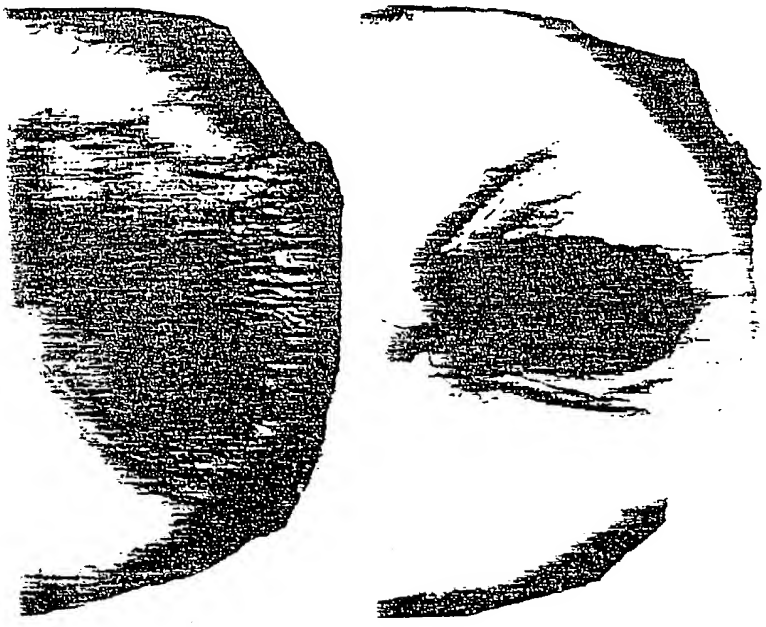
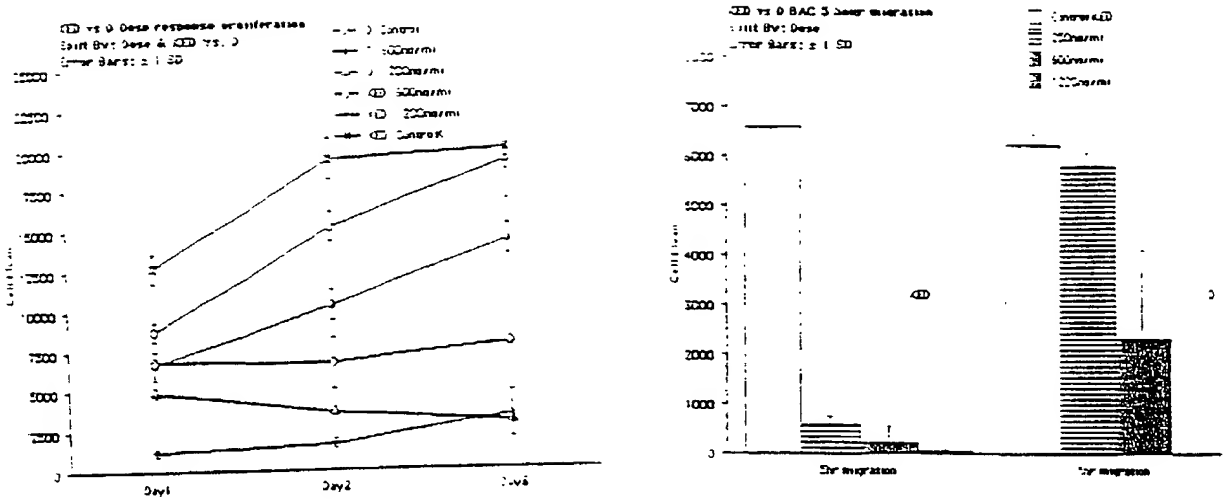


Fig. 19



C





A B

Fig. 20

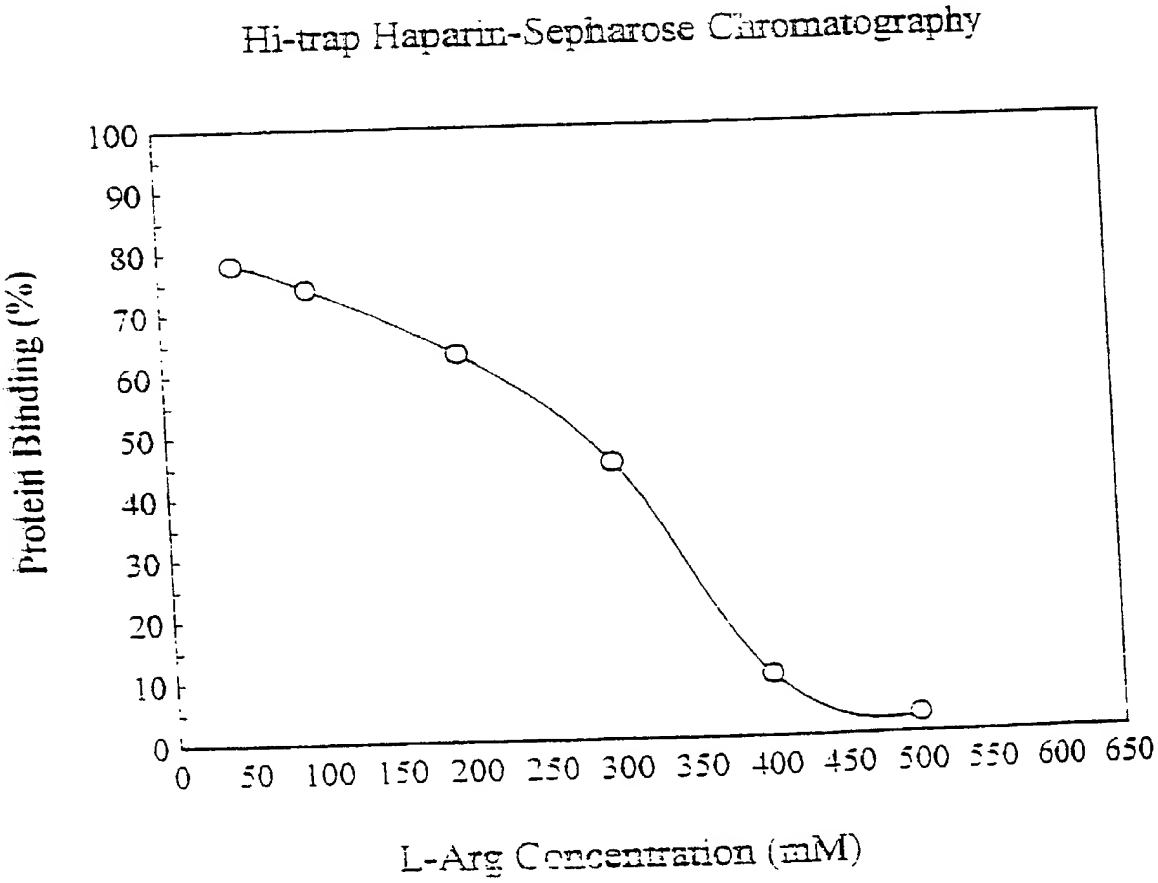
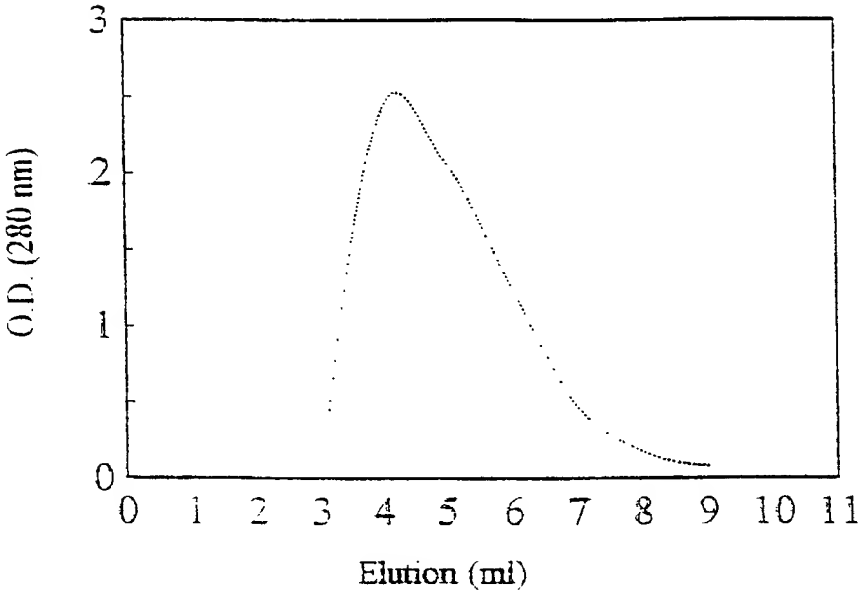


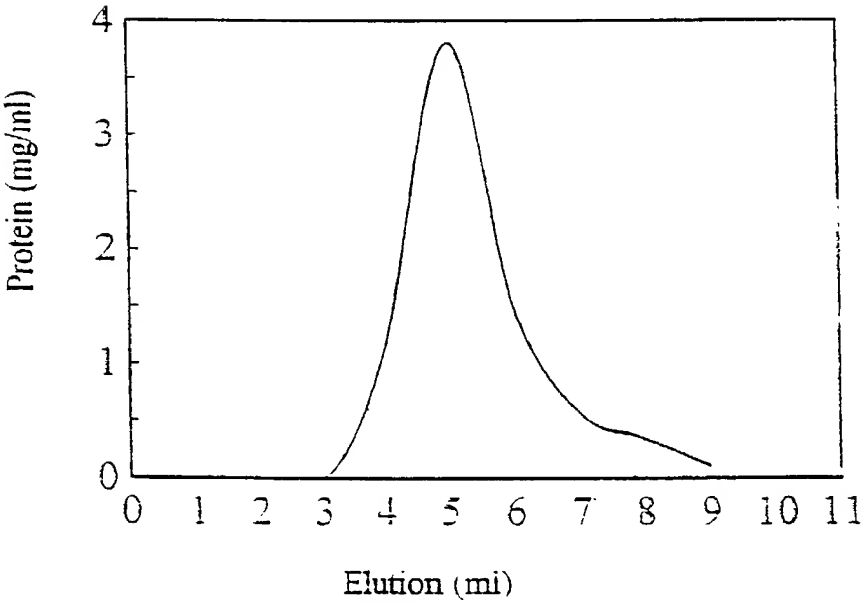
Fig. 21 A

Fig. 21

Hi-trap Heparin-Sepharose Chromatography (5ml)
Loading buffer: 10 mM Tris (pH7.4).
Elution buffer: 10 mM Tris (pH7.4) containing 1 M NaCl



B



C

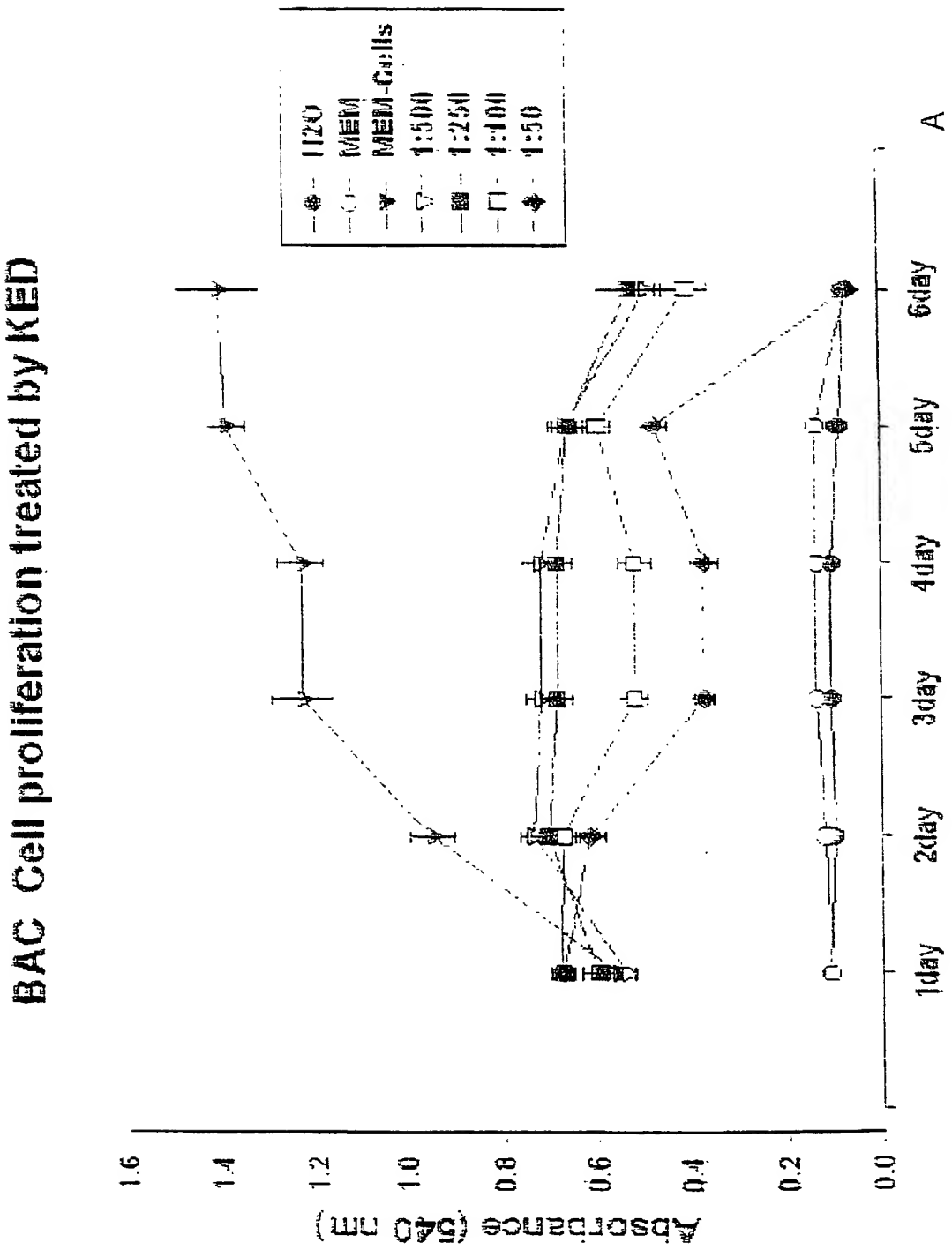

BAC Cell proliferation treated by KED
1120
MEM
MEM-Cells
1:500
1:250
1:100
1:50
Absorbance (540 nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1day
2day
3day
4day
5day
6day
A

Fig. 22

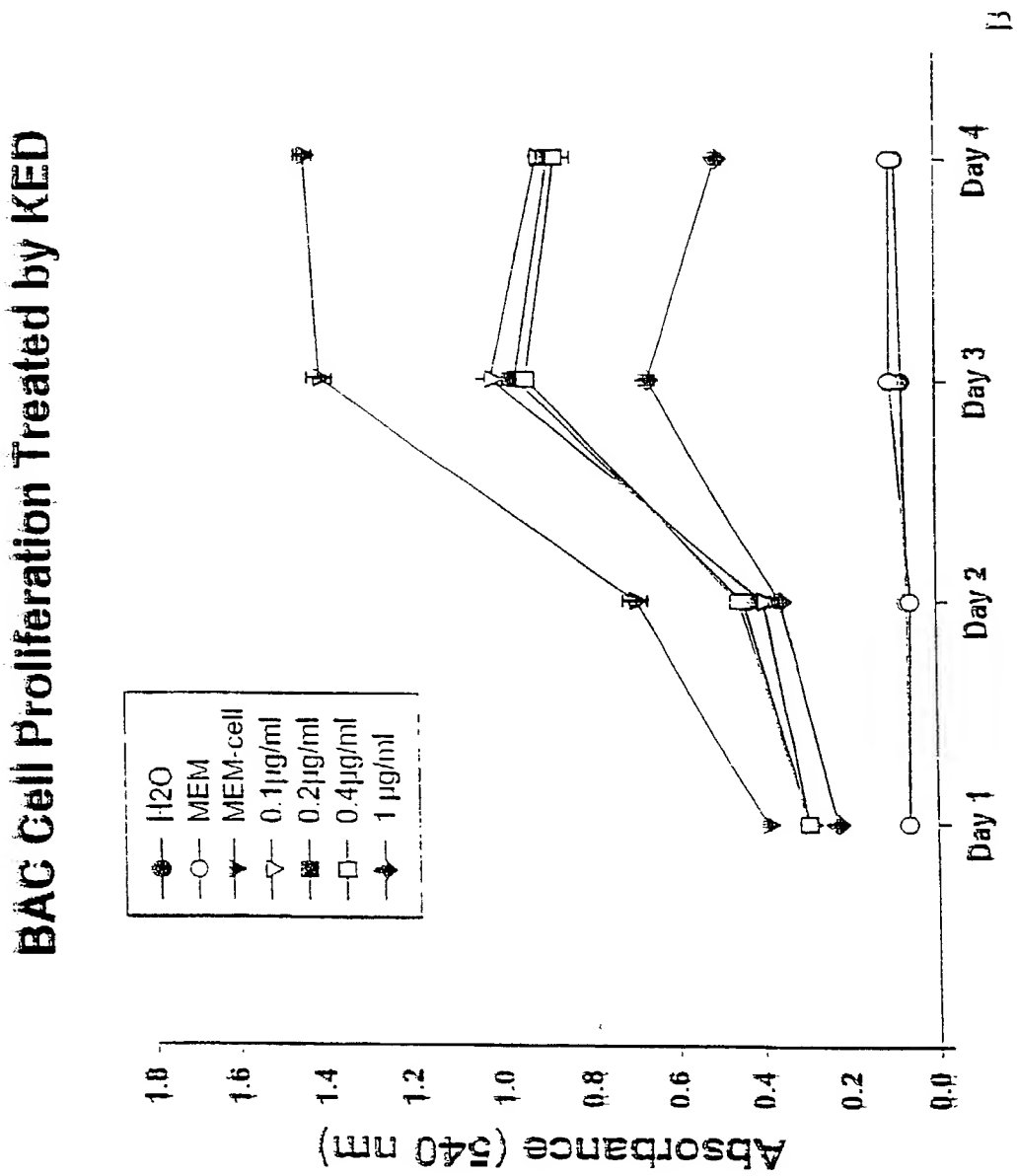

BAC Cell Proliferation Treated by KED
H2O
MEM
MEM-cell
0.1µg/ml
0.2µg/ml
0.4µg/ml
1 µg/ml
Absorbance (540 nm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Day 1
Day 2
Day 3
Day 4

Fig. 22

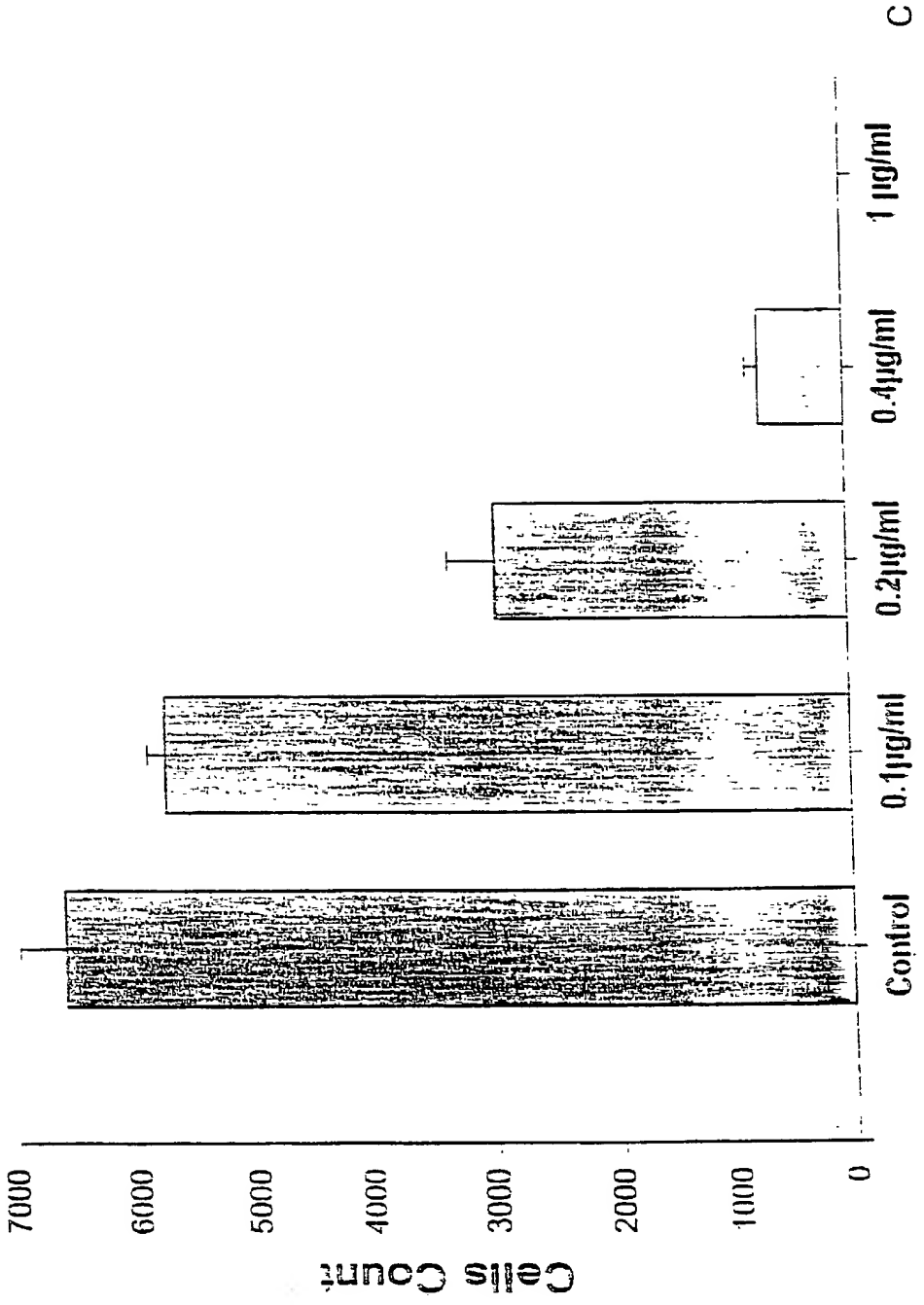

BAC Cell Migration Treated by KED
Cells Count
7000
6000
5000
4000
3000
2000
1000
0
Control
0.1μg/ml
0.2μg/ml
0.4μg/ml
1 μg/ml
C

Fig. 22

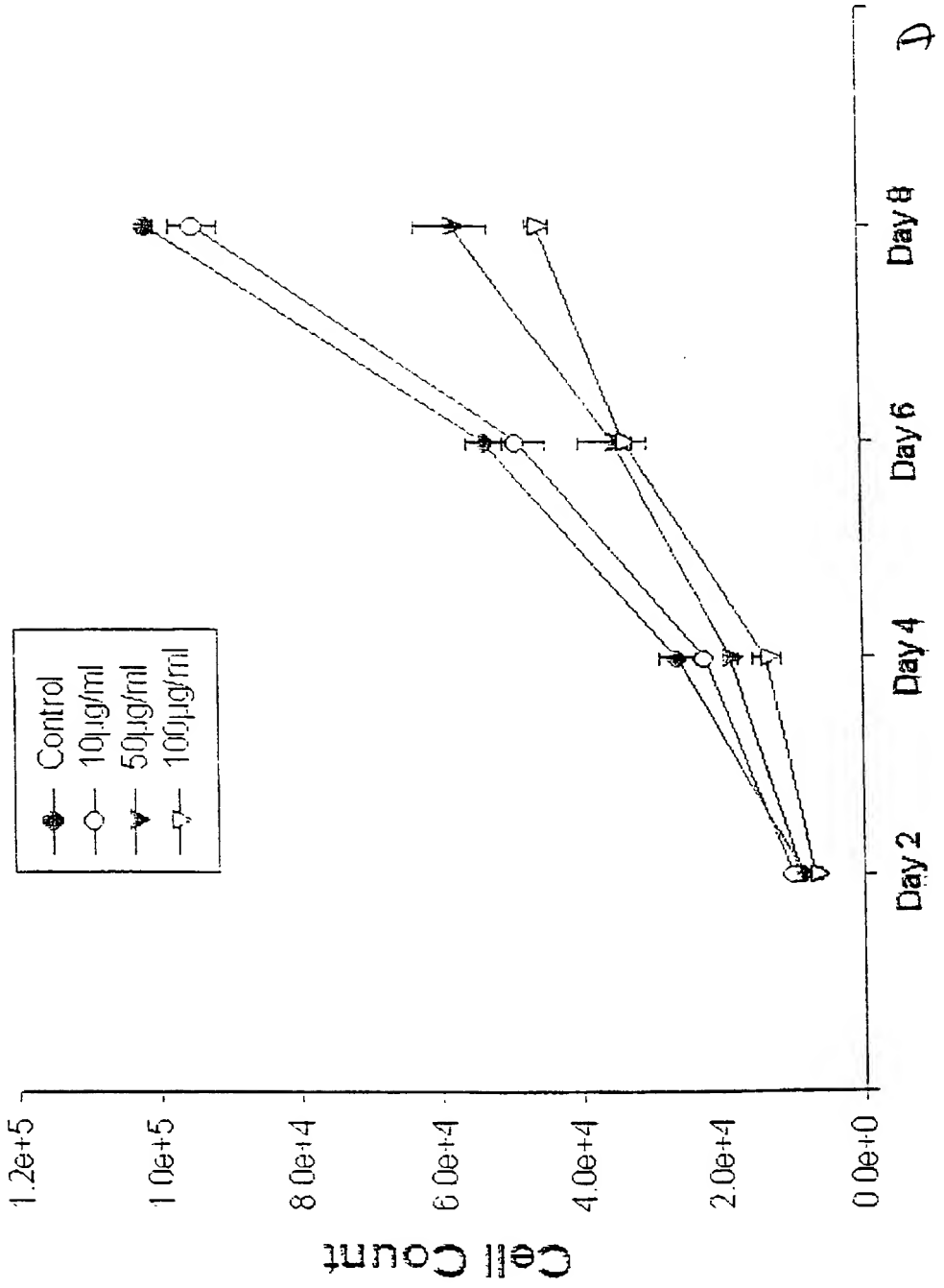

BAC Cell Proliferation Treated by Angiostatin
Cell Count
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0e+0
Day 2
Day 4
Day 6
Day 8
Control
10μg/ml
50μg/ml
100μg/ml

Fig. 22

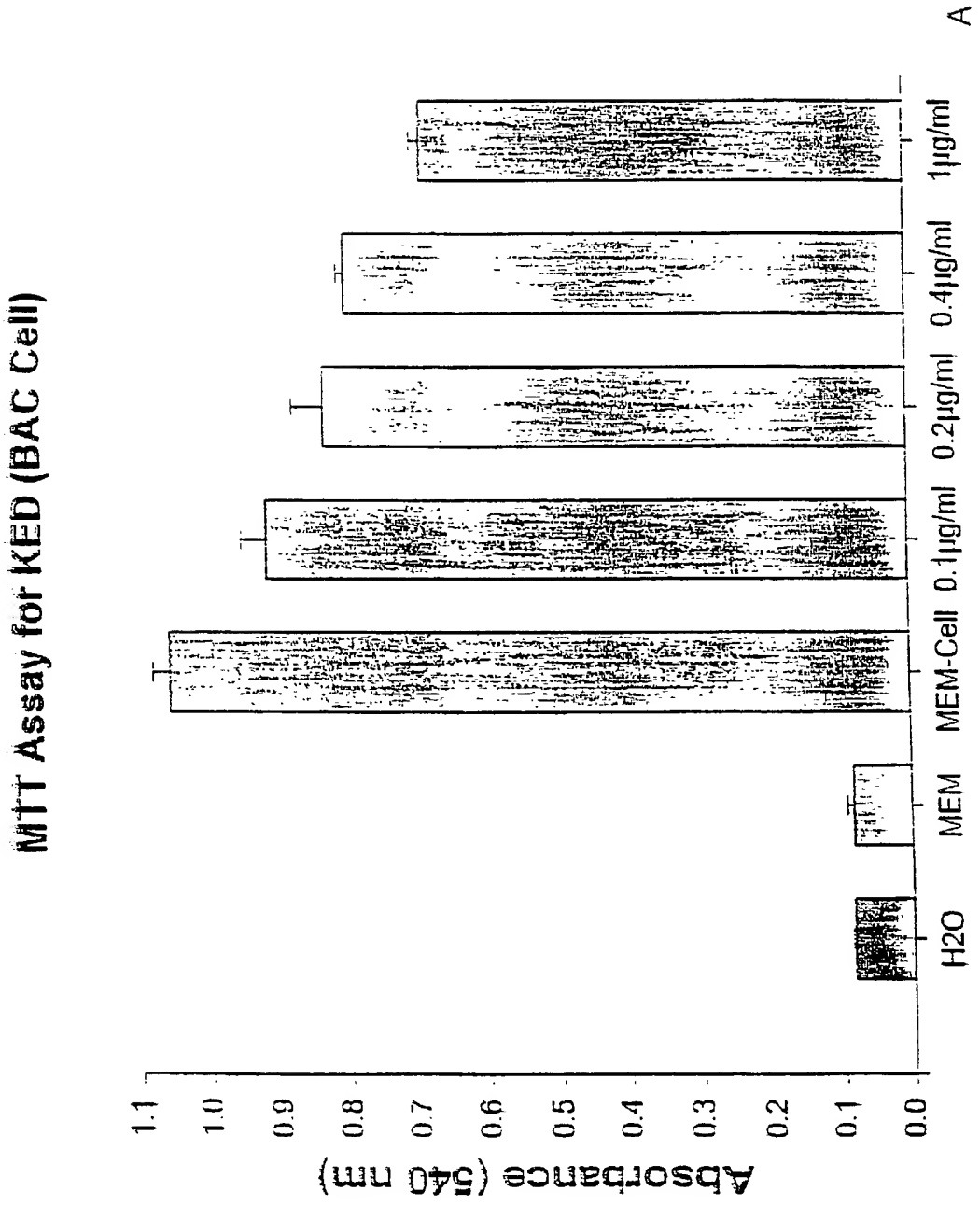

MTT Assay for KED (BAC Cell)
Absorbance (540 nm)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
H2O
MEM
MEM-Cell
0.1µg/ml
0.2µg/ml
0.4µg/ml
1µg/ml
A

Fig. 23

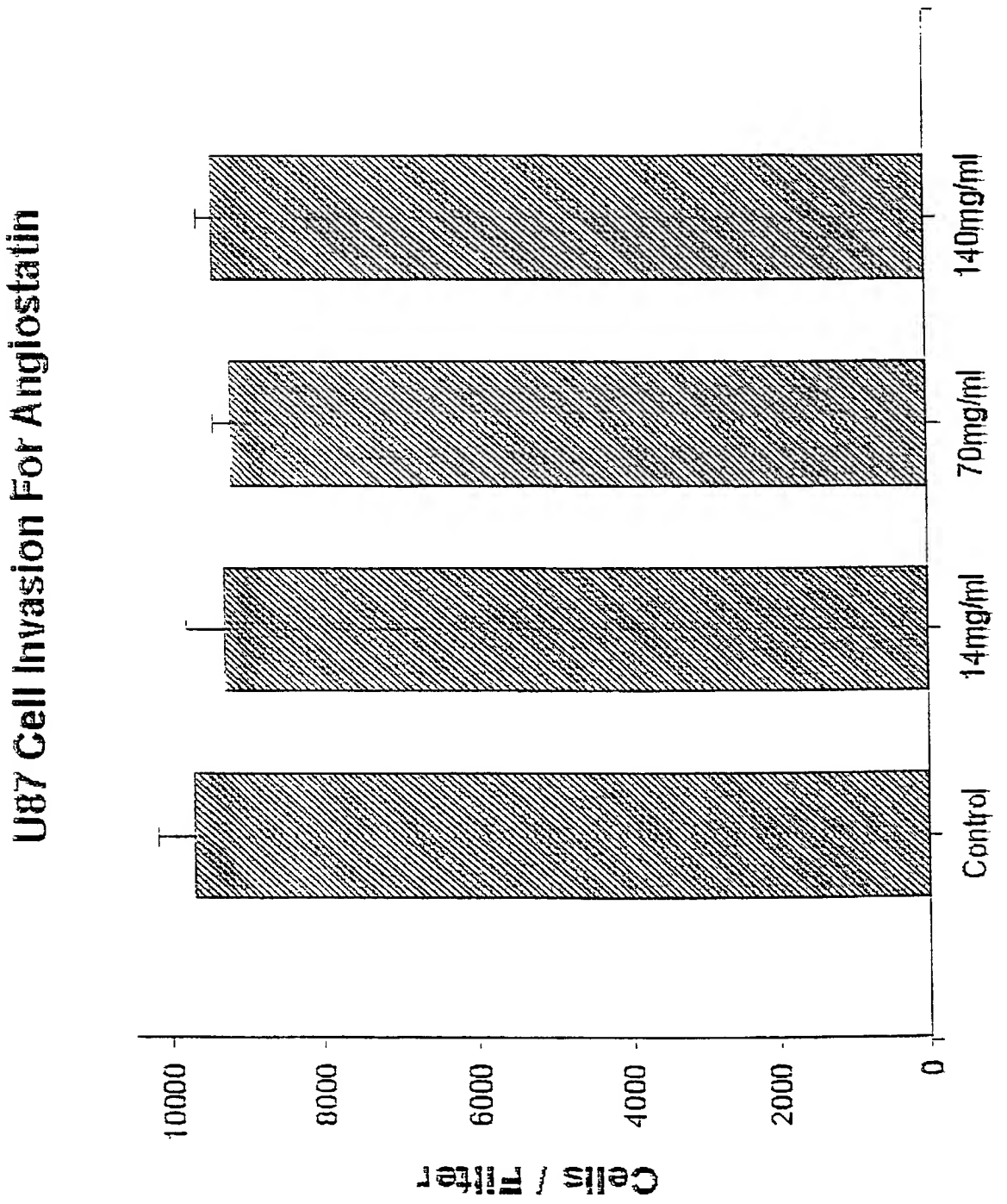

U87 Cell Invasion For Angiostatin
Cells / Filter
10000
8000
6000
4000
2000
0
Control
14mg/ml
70mg/ml
140mg/ml
B

Fig. 23

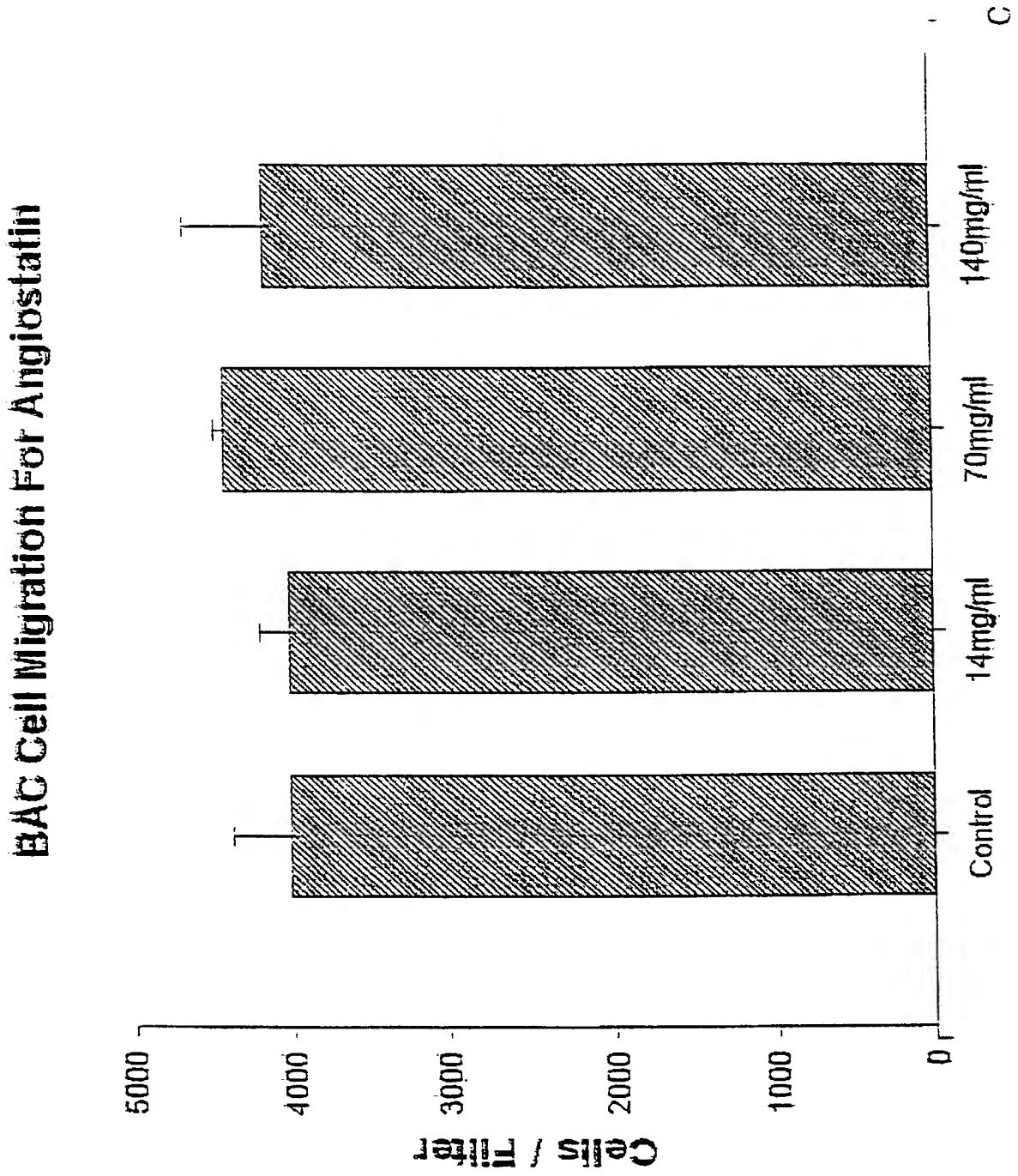
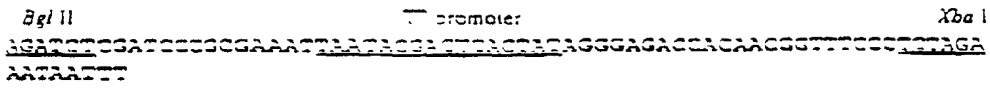

BAC Cell Migration For Angiostatin
Cells / Filter
5000
4000
3000
2000
1000
0
Control
14mg/ml
70mg/ml
140mg/ml
C

Fig. 23

pET-3a-d



pET-11a-d

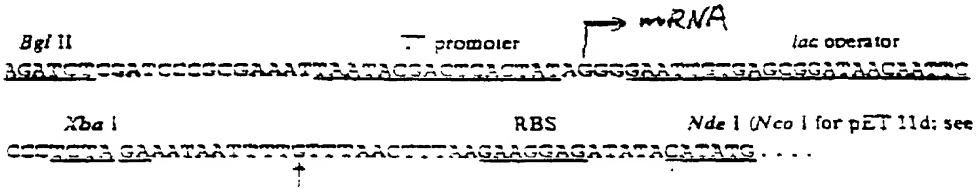


Figure 1 The pET system promoters and mRNA leader sequences.

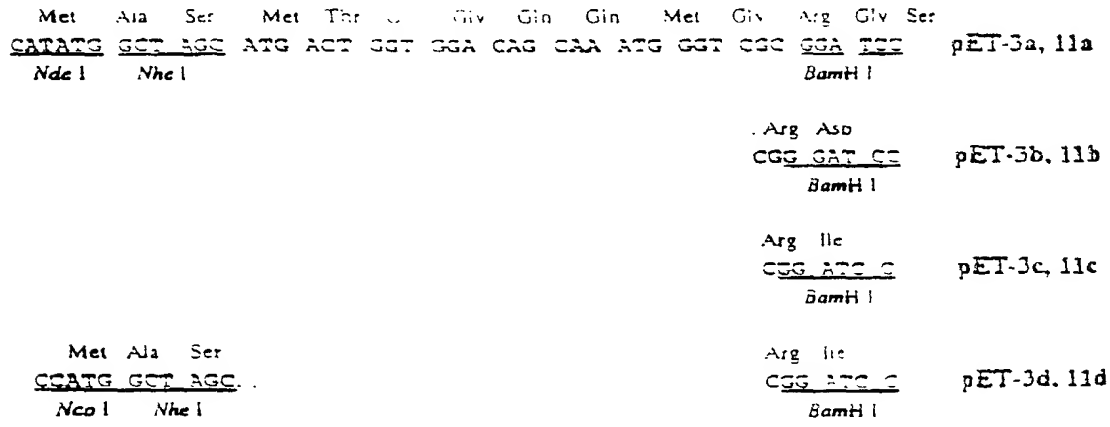
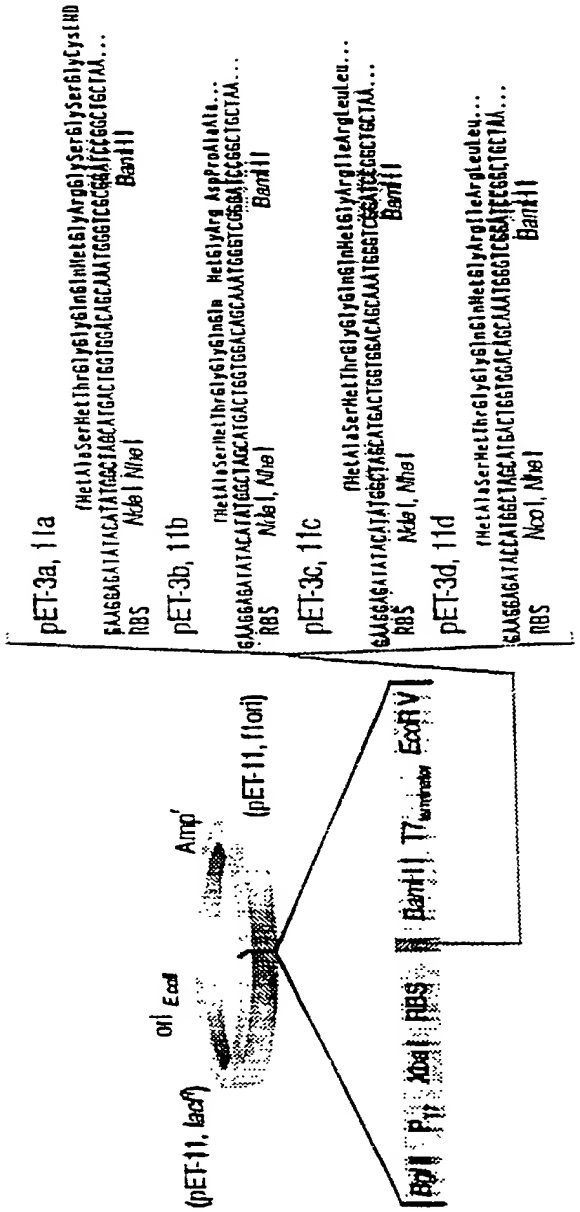


Figure 2 The pET system cloning sites.

A

Fig. 2A


pET-3a, 11a
pET-3b, 11b
pET-3c, 11c
pET-3d, 11d
Amp'
(pET-11, f1ori)
ori
(pET-11, lacI)
BamH I
T7 terminator
EcoR V
Pst I
Xba I
RBS
Nde I, Nhe I
Nde I
Nco I, Nhe I
BamH I

B

Fig. 24

XED

(A)

```
          10         20         30         40         50         60
atggaagaag actgtatgtt tgggaatggg aaaggatacc gaggcaagag ggcgaccact
          70         80         90        100        110        120
gttactggga cgccatgcca ggactgggct gcccaggagc cccatagaca cagcatttte
         130        140        150        160        170        180
acttccagaga caaatccacg ggcgggtctg gaaaaaaatt actgccgtaa ccctgatggt
         190        200        210        220        230        240
gatgtaggtg gtccctggtg ctacacgaca aatccaagaa aactttacga ctactgtgat
         250        260        270        280        290        300
gtccctcagt gtgcggccca tatgcacagc caccgcgact tccagccggt gctccacctg
         310        320        330        340        350        360
gttgcgctca acagcccctt gtcaggcggc atgcggggca tccgcggggc cgacttccag
         370        380        390        400        410        420
tgcttccagc aggcgcgggc cgtggggctg gcgggcacct tccgcgcctt cctgtcctcg
         430        440        450        460        470        480
cgcctgcagg acctgtacag catcgtgcgc cgtgccgacc gcgcagccgt gcccatcgtc
         490        500        510        520        530        540
aacctcaagg acgagctgct gtttcccagc tgggaggctc tgttctcagg ctctgagggt
         550        560        570        580        590        600
ccgctgaagc ccggggcacg catcttctcc tttgacggca aggacgtcct gaggcacccc
         610        620        630        640        650        660
acctggcccc agaagagcgt gtggcatggc tcggacccca acgggcgcag gctgaccgag
         670        680        690        700        710        720
agctactgtg agacgtggcg gacggaggct ccctcggcca cgggccaggc ctcctcgctg
         730        740        750        760        770        780
ctgggggcca ggctcctggg gcagagtgcc gcgagctgcc atcacgccta catcgtgctc
         790        800        810        820        830        840
tgcattgaga acagcttcat gactgcctcc aagtag.... .......... ..........
```

XED

(B)

```
          10         20         30         40         50         60
MEEDCMFGNG KGYRGKRATT VTGTPCQDWA AQEPHRHSIF TPETNPRAGL EKNYCRNPDG
          70         80         90        100        110        120
DVGGPWCYTT NPRKLYDYCD VPQCAAHMHS HRDFQPVLHL VALNSPLSGG MRGIRGADFQ
         130        140        150        160        170        180
CFQQARAVGL AGTFRAFLSS RLQDLYSIVR RADRAAVPIV NLKDELLFPS WEALFSGSEG
         190        200        210        220        230        240
PLKPGARIFS FDGKDVLRHP TWPQKSVWHG SDPNGRRLTE SYCETWRTEA PSATGQASSL
         250        260        270        280        290        300
LGGRLLGQSA ASCHHAYIVL CIENSFMTAS K*.....  ..........  ..........
```

SEQUENCE LISTING

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AATGGTTTCT TAGACGTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA CCCCTATTTG
       130         140         150         160         170         180
TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAT
       190         200         210         220         230         240
GCTTCAATAA TATTGAAAAA GGAAGAGTAT GAGTATTCAA CATTTCCGTG TCGCCCTTAT
       250         260         270         280         290         300
TCCCTTTTTT GCGGCATTTT GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT
       310         320         330         340         350         360
AAAAGATGCT GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG
       370         380         390         400         410         420
CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA GCACTTTTAA
       430         440         450         460         470         480
AGTTCTGCTA TGTGGCGCGG TATTATCCCG TGTTGACGCC GGGCAAGAGC AACTCGGTCG
       490         500         510         520         530         540
CCGCATACAC TATTCTCAGA ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT
       550         560         570         580         590         600
TACGGATGGC ATGACAGTAA GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC
       610         620         630         640         650         660
TGCGGCCAAC TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA
       670         680         690         700         710         720
CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT
       730         740         750         760         770         780
ACCAAACGAC GAGCGTGACA CCACGATGCC TGCAGCAATG GCAACAACGT TGCGCAAACT
       790         800         810         820         830         840
ATTAACTGGC GAACTACTTA CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC
       850         860         870         880         890         900
GGATAAAGTT GCAGGACCAC TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA
       910         920         930         940         950         960
TAAATCTGGA GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG
       970         980         990        1000        1010        1020
TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG
      1030        1040        1050        1060        1070        1080
AAATAGACAG ATCGCTGAGA TAGGGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA
      1090        1100        1110        1120        1130        1140
GTTTACTCAT ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA AAGGATCTAG
      1150        1160        1170        1180        1190        1200
GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT TTCGTTCCAC
      1210        1220        1230        1240        1250        1260
TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT TTTTCTGCGC
      1270        1280        1290        1300        1310        1320
GTAATCTGCT GCTTGCAAAC AAAAAAACCA CCGCTACCAG CGGTGGTTTG TTTGCCGGAT
      1330        1340        1350        1360        1370        1380
CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT
      1390        1400        1410        1420        1430        1440
ACTGTCCTTC TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT
      1450        1460        1470        1480        1490        1500
```

pET vector

ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA TAAGTCGTGT
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1570 1580 1590 1600 1610 1620
GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT ACACCGAACT GAGATACCTA
1630 1640 1650 1660 1670 1680
CAGCGTGAGC TATGAGAAAG CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG
1690 1700 1710 1720 1730 1740
GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG AAACGCCTGG
1750 1760 1770 1780 1790 1800
TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT TTTGTGATGC
1810 1820 1830 1840 1850 1860
TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCAACG CGGCCTTTTT ACGGTTCCTG
1870 1880 1890 1900 1910 1920
GCCTTTTGCT GGCCTTTTGC TCACATGTTC TTTCCTGCGT TATCCCCTGA TTCTGTGGAT
1930 1940 1950 1960 1970 1980
AACCGTATTA CCGCCTTTGA GTGAGCTGAT ACCGCTCGCC GCAGCCGAAC GACCGAGCGC
1990 2000 2010 2020 2030 2040
AGCGAGTCAG TGAGCGAGGA AGCGGAAGAG CGCCTGATGC GGTATTTTCT CCTTACGCAT
2050 2060 2070 2080 2090 2100
CTGTGCGGTA TTTCACACCG CATATATGGT GCACTCTCAG TACAATCTGC TCTGATGCCG
2110 2120 2130 2140 2150 2160
CATAGTTAAG CCAGTATACA CTCCGCTATC GCTACGTGAC TGGGTCATGG CTGCGCCCCG
2170 2180 2190 2200 2210 2220
ACACCCGCCA ACACCCGCTG ACGCGCCCTG ACGGGCTTGT CTGCTCCCGG CATCCGCTTA
2230 2240 2250 2260 2270 2280
CAGACAAGCT GTGACCGTCT CCGGGAGCTG CATGTGTCAG AGGTTTTCAC CGTCATCACC
2290 2300 2310 2320 2330 2340
GAAACGCGCG AGGCAGCTGC GGTAAAGCTC ATCAGCGTGG TCGTGAAGCG ATTCACAGAT
2350 2360 2370 2380 2390 2400
GTCTGCCTGT TCATCCGCGT CCAGCTCGTT GAGTTTCTCC AGAAGCGTTA ATGTCTGGCT
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TCTGATAAAG CGGGCCATGT TAAGGGCGGT TTTTTCCTGT TTGGTCACTG ATGCCTCCGT
2470 2480 2490 2500 2510 2520
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2530 2540 2550 2560 2570 2580
GATACGGGTT ACTGATGATG AACATGCCCG GTTACTGGAA CGTTGTGAGG GTAAACAACT
2590 2600 2610 2620 2630 2640
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2650 2660 2670 2680 2690 2700
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2710 2720 2730 2740 2750 2760
CATAATGGTG CAGGGCGCTG ACTTCCGCGT TTCCAGACTT TACGAAACAC GGAAACCGAA
2770 2780 2790 2900 2810 2920
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2930 2840 2850 2960 2870 2880
CTCGCGTATC GGTGATTCAT TCTGCTAACC AGTAAGGCAA CCCCGCCAGC CTAGCCGGGT
2990 2900 2910 2920 2930 2940
CCTCAACGAC AGGAGCACGA TCATGCGCAC CCGTGGCCAG GACCCAACGC TGCCCGAGAT

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      2950        2960        2970        2980        2990        3000
GCGCCGCGTG CGGCTGCTGG AGATGGCGGA CGCGATGGAT ATGTTCTGCC AAGGGTTGGT
      3010        3020        3030        3040        3050        3060
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      3070        3080        3090        3100        3110        3120
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      3130        3140        3150        3160        3170        3180
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      3190        3200        3210        3220        3230        3240
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      3250        3260        3270        3280        3290        3300
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      3310        3320        3330        3340        3350        3360
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      3430        3440        3450        3460        3470        3480
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      3550        3560        3570        3580        3590        3600
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      3610        3620        3630        3640        3650        3660
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      3850        3860        3870        3880        3890        3900
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      4150        4160        4170        4180        4190        4200
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      4210        4220        4230        4240        4250        4260
CTATCGGCTG AATTTGATTG CGAGTGAGAT ATTTATGCCA GCCAGCCAGA CGCAGACGCG
      4270        4280        4290        4300        4310        4320
CCGAGACAGA ACTTAATGGG CCCGCTAACA GCGCGATTTG CTGGTGACCC AATGCGACCA
      4330        4340        4350        4360        4370        4380
GATGCTCCAC GCCCAGTCGC GTACCGTCTT CATGGGAGAA AATAATACTG TTGATGGGTG
      4390        4400        4410        4420        4430        4440
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4510 4520 4530 4540 4550 4560
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4570 4580 4590 4600 4610 4620
CGCTGGCACC CAGTTGATCG GCGCGAGATT TAATCGCCGC GACAATTTGC GACGGCGCGT
4630 4640 4650 4660 4670 4680
GCAGGGCCAG ACTGGAGGTG GCAACGCCAA TCAGCAACGA CTGTTTGCCC GCCAGTTGTT
4690 4700 4710 4720 4730 4740
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4750 4760 4770 4780 4790 4800
TTTTCGCAGA AACGTGGCTG GCCTGGTTCA CCACGCGGGA AACGGTCTGA TAAGAGACAC
4810 4820 4830 4840 4850 4860
CGGCATACTC TGCGACATCG TATAACGTTA CTGGTTTCAC ATTCACCACC CTGAATTGAC
4870 4880 4890 4900 4910 4920
TCTCTTCCGG GCGCTATCAT GCCATACCGC GAAAGGTTTT GCGCCATTCG ATGGTGTCCG
4930 4940 4950 4960 4970 4980
GGATCTCGAC GCTCTCCCTT ATGCGACTCC TGCATTAGGA AGCAGCCCAG TAGTAGGTTG
4990 5000 5010 5020 5030 5040
AGGCCGTTGA GCACCGCCGC CGCAAGGAAT GGTGCATGCA AGGAGATGGC GCCCAACAGT
5050 5060 5070 5080 5090 5100
CCCCCGGCCA CGGGGCCTGC CACCATACCC ACGCCGAAAC AAGCGCTCAT GAGCCCGAAG
5110 5120 5130 5140 5150 5160
TGGCGAGCCC GATCTTCCCC ATCGGTGATG TCGGCGATAT AGGCGCCAGC AACCGCACCT
5170 5180 5190 5200 5210 5220
GTGGCGCCGG TGATGCCGGC CACGATGCGT CCGGCGTAGA GGATCGAGAT CTCGATCCCG
5230 5240 5250 5260 5270 5280
CGAAATTAAT ACGACTCACT ATAGGGGAAT TGTGAGCGGA TAACAATTCC CCTCTAGAAA
5290 5300 5310 5320 5330 5340
TAATTTTGTT TAACTTTAAG AAGGAGATAT AcatatgAAC AGTGACTGCT ACTTTGGGAA
5350 5360 5370 5380 5390 5400
TGGGTCAGCC TACCGTGGCA CGCACAGCCT CACCGAGTCG GGTGCCTCCT GCCTCCCGTG
5410 5420 5430 5440 5450 5460
GAATTCCATG ATCCTGATAG GCAAGGTTTA CACAGCACAG AACCCCAGTG CCCAGGCACT
5470 5480 5490 5500 5510 5520
GGGCCTGGGC AAACATAATT ACTGCCGGAA TCCTGATGGG GATGCCAAGC CCTGGTGCta
5530 5540 5550 5560 5570 5580
tGTGCTGAAG AACCGCAGGC TGACGTGGGA GTACTGTGAT GTGCCCTCCT GCTCCACCca
5590 5600 5610 5620 5630 5640
tCACCATcac CATcaccgcg acttccagcc ggtgctccac ctggttgcgc tcaacagccc
5650 5660 5670 5680 5690 5700
cctgtcaggc ggcatgcggg gcatccgcgg ggccgacttc cagtgctttc agcaggcgcg
5710 5720 5730 5740 5750 5760
ggccgtgggg ctggcgggca ccttccgcgc ctttctgtcc tcgcgcctgc aggacctgta
5770 5780 5790 5800 5810 5820
cagcatcgtg cgccgtgccg accgccagc cgtgcccatc gttaacctca aggacgagct
5830 5840 5850 5860 5870 5880
gctgtttccc agctgggagg ctttgttccc aggctctgag ggtcccgctga agccccgggc

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      5890       5900       5910       5920       59..       5940
acgcatcttc tcctttgacg gcaaggacgt cctgaggcac cccacctggc cccagaagag
      5950       5960       5970       5980       5990       6000
cgtgtggcat ggctcggacc ctaacggcgg caggctgacc gagagctact gtgagacgtg
      6010       6020       6030       6040       6050       6060
gcggacggag gctccccteg ccacggccca ggcctcctcg ctgctggggg gcaggctcct
      6070       6080       6090       6100       6110       6120
ggggcagagt gccgcgagct gccatcacgc ctacatcgtg ctctgcattg agaacagctt
      6130       6140       6150       6160       6170       6180
catgactgcc tccaagtaGG ATCCGGCTGC TAACAAAGCC CGAAAGGAAG CTGAGTTGGC
      6190       6200       6210       6220       6230       6240
TGCTGCCACC GCTGAGCAAT AACTAGCATA ACCCCTTGGG GCCTCTAAAC GGGTCTTGAG
      6250       6260       6270       6280       6290       6300
GGGTTTTTTG CTGAAAGGAG GAACTATATC CGGATATCCC GCAAGAGGCC CGGCAGTACC
      6310       6320       6330       6340       6350       6360
GGCATAACCA AGCCTATGCC TACAGCATCC AGGGTGACGG TGCCGAGGAT GACGATGAGC
      6370       6380       6390       6400       6410       6420
GCATTGTTAG ATTTCATACA CGGTGCCTGA CTGCGTTAGC AATTTAACTG TGATAAACTA
      6430       6440       6450       6460       6470       6480
CCGCATTAAA GCTTATCGAT GATAAGCTGT CAAACATGAG AA........ ..........
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Sequence of pET vector 3a.

```
                10        20        30        40        50        60
                |         |         |         |         |         |
   1 TTCTCATGTT TGACAGCTTA TCATCGATAA GCTTTAATGC GGTAGTTTAT CACAGTTAAA
  61 TTGCTAACGC AGTCAGGCAC CGTGTATGAA ATCTAACAAT GCGCTCATCG TCATCCTCGG
 121 CACCGTCACC CTGGATGCTG TAGGCATAGG CTTGGTTATG CCGGTACTGC CGGGCCTCTT
 181 GCGGGATATC GTCCATTCCG ACAGCATCGC CAGTCACTAT GGCGTGCTGC TAGCGCTATA
 241 TGCGTTGATG CAATTTCTAT GCGCACCCGT TCTCGGAGCA CTGTCCGACC GCTTTGGCCG
 301 CCGCCCAGTC CTGCTCGCTT CGCTACTTGG AGCCACTATC GACTACGCGA TCATGGCGAC
 361 CACACCCGTC CTGTGGATAT CCGGATATAG TTCCTCCTTT CAGCAAAAAA CCCCTCAAGA
 421 CCCGTTTAGA GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAACT
 481 CAGCTTCCTT TCGGGCTTTG TTAGCAGCCG GATCGCGAC CCATTTGCTG TCCACCAGTC
 541 ATGCTAGCCA TATGTATATC TCCTTCTTAA AGTTAAACAA AATTATTTCT AGAGGGAAAC
 601 CGTTGTGGTC TCCCTATAGT GAGTCGTATT AATTTCGCGG GATCGAGATC TCGATCCTCT
 661 ACGCCGGACG CATCGTGGCC GGCATCACCG GCGCCACAGG TGCGGTTGCT GGCGCCTATA
 721 TCGCCGACAT CACCGATGGG GAAGATCGGG CTCGCCACTT CGGGCTCATG AGCGCTTGTT
 781 TCGGCGTGGG TATGGTGGCA GGCCCCGTGG CCGGGGGACT GTTGGGCGCC ATCTCCTTGC
 841 ATGCACCATT CCTTGCGGCG GCGGTGCTCA ACGGCCTCAA CCTACTACTG GGCTGCTTCC
 901 TAATGCAGGA GTCGCATAAG GGAGAGCGTC GACCGATGCC CTTGAGAGCC TTCAACCCAG
 961 TCAGCTCCTT CCGGTGGGCG CGGGGCATGA CTATCGTCGC CGCACTTATG ACTGTCTTCT
1021 TTATCATGCA ACTCGTAGGA CAGGTGCCGG CAGCGCTCTG GGTCATTTTC GGCGAGGACC
1081 GCTTTCGCTG GAGCGCGACG ATGATCGGCC TGTCGCTTGC GGTATTCGGA ATCTTGCACG
1141 CCCTCGCTCA AGCCTTCGTC ACTGGTCCCG CCACCAAACG TTTCGGCGAG AAGCAGGCCA
1201 TTATCGCCGG CATGGCGGCC GACGCGCTGG GCTACGTCTT GCTGGCGTTC GCGACGCGAG
1261 GCTGGATGGC CTTCCCCATT ATGATTCTTC TCGCTTCCGG CGGCATCGGG ATGCCCGCGT
1321 TGCAGGCCAT GCTGTCCAGG CAGGTAGATG ACGACCATCA GGGACAGCTT CAAGGATCGC
1381 TCGCGGCTCT TACCAGCCTA ACTTCGATCA CTGGACCGCT GATCGTCACG GCGATTTATG
1441 CCGCCTCGGC GAGCACATGG AACGGGTTGG CATGGATTGT AGGCGCCGCC CTATACCTTG
1501 TCTGCCTCCC CGCGTTGCGT CGCGGTGCAT GGAGCCGGGC CACCTCGACC TGAATGGAAG
1561 CCGGCGGCAC CTCGCTAACG GATTCACCAC TCCAAGAATT GGAGCCAATC AATTCTTGCG
1621 GAGAACTGTG AATGCGCAAA CCAACCCTTG GCAGAACATA TCCATCGCGT CCGCCATCTC
1681 CAGCAGCCGC ACGCGGCGCA TCTCGGGCAG CGTTGGGTCC TGGCCACGGG TGCGCATGAT
1741 CGTGCTCCTG TCGTTGAGGA CCCGGCTAGG CTGGCGGGGT TGCCTTACTG GTTAGCAGAA
1801 TGAATCACCG ATACGCGAGC GAACGTGAAG CGACTGCTGC TGCAAAACGT CTGCGACCTG
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1861 AGCAACAACA TGAATGGTCT TCGGTTTCCG TGTTTCGTAA AGTCTGGAAA CGCGGAAGTC
1921 AGCGCCCTGC ACCATTATGT TCCGGATCTG CATCGCAGGA TGCTGCTGGC TACCCTGTGG
1981 AACACCTACA TCTGTATTAA CGAAGCGCTG GCATTGACCC TGAGTGATTT TTCTCTGGTC
2041 CCGCCGCATC CATACCGCCA GTTGTTTACC CTCACAACGT TCCAGTAACC GGGCATGTTC
2101 ATCATCAGTA ACCCGTATCG TGAGCATCCT CTCTCGTTTC ATCGGTATCA TTACCCCCAT
2161 GAACAGAAAT CCCCCTTACA CGGAGGCATC AGTGACCAAA CAGGAAAAAA CCGCCCTTAA
2221 CATGGCCCGC TTTATCAGAA GCCAGACATT AACGCTTCTG GAGAAACTCA ACGAGCTGGA
2281 CGCGGATGAA CAGGCAGACA TCTGTGAATC GCTTCACGAC CACGCTGATG AGCTTTACCG
2341 CAGCTGCCTC GCGCGTTTCG GTGATGACGG TGAAAACCTC TGACACATGC AGCTCCCGGA
2401 GACGGTCACA GCTTGTCTGT AAGCGGATGC CGGGAGCAGA CAAGCCCGTC AGGGCGCGTC
2461 AGCGGGTGTT GGCGGGTGTC GGGGCGCAGC CATGACCCAG TCACGTAGCG ATAGCGGAGT
2521 GTATACTGGC TTAACTATGC GGCATCAGAG CAGATTGTAC TGAGAGTGCA CCATATATGC
2581 GGTGTGAAAT ACCGCACAGA TGCGTAAGGA GAAAATACCG CATCAGGCGC TCTTCCGCTT
2641 CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA TCAGCTCACT
2701 CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG
2761 CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA
2821 GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC
2881 CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG
2941 TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC
3001 TTTCTCATAG CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG
3061 GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC
3121 TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA
3181 TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG
3241 GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA
3301 AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG
3361 TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT
3421 CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT
3481 TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT
3541 AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA
3601 TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG ACTCCCCGTC GTGTAGATAA
3661 CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC
3721 GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA
3781 GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG

Cont. Sequence of pET vector 3a.

3841 TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTGCA GGCATCGTGG
3901 AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCC
2101 ATCATCAGTA ACCCGTATCG TGAGCATCCT CTCTCGTTTC ATCGGTATCA TTACCCCCAT
2161 GAACAGAAAT CCCCCTTACA CGGAGGCATC AGTGACCAAA CAGGAAAAAA CCGCCCTTAA
2221 CATGGCCCGC TTTATCAGAA GCCAGACATT AACGCTTCTG GAGAAACTCA ACGAGCTGGA
2281 CGCGGATGAA CAGGCAGACA TCTGTGAATC GCTTCACGAC CACGCTGATG AGCTTTACCG
2341 CAGCTGCCTC GCGCGTTTCG GTGATGACGG TGAAAACCTC TGACACATGC AGCTCCCGGA
2401 GACGGTCACA GCTTGTCTGT AAGCGGATGC CGGGAGCAGA CAAGCCCGTC AGGGCGCGTC
2461 AGCGGGTGTT GGCGGGTGTC GGGGCGCAGC CATGACCCAG TCACGTAGCG ATAGCGGAGT
2521 GTATACTGGC TTAACTATGC GGCATCAGAG CAGATTGTAC TGAGAGTGCA CCATATATGC
2581 GGTGTGAAAT ACCGCACAGA TGCGTAAGGA GAAAATACCG CATCAGGCGC TCTTCCGCTT
2641 CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA TCAGCTCACT
2701 CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG
2761 CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA
2821 GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC
2981 CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG
2941 TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC
3001 TTTCTCATAG CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG
3061 GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC
3121 TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA
3181 TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG
3241 GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA
3301 AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG
3361 TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT
3421 CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT
3481 TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT
3541 AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA
3601 TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG ACTCCCCGTC GTGTAGATAA
3661 CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC
3721 GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA
3781 GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG
3841 TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTGCA GGCATCGTGG
3901 TGTCACGCTC GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG
3961 TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG

4021 TCAGAAGTAA GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC
4081 TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT
4141 TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC GGCGTCAACA CGGGATAATA
4201 CCGCGCCACA TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA
4261 AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA
4321 ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC
4381 AAAATGCCGC AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC
4441 TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG
4501 AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC
4561 CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA
4621 GGCCCTTTCG TCTTCAAGAA

Total number of bases is 4640.
DNA sequence composition: 1050 A, 1281 C, 1194 G and 1115 T.

Sequence of pET vector 3a.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Full name of fifth inventor, if any

Fifth inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of sixth inventor, if any

Sixth inventor's signature Date

Residence

Citizenship

Post Office Address

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/121,341	02/24/99
(Application Serial No.)	(Filing Date)
60/121,633	02/25/99
(Application Serial No.)	(Filing Date)
60/166,176	11/18/99
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/US00/04798	02/24/00	pending
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Docket No.
1059.00051

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
ANTI-ANGIOGENIC KRINGLE PROTEIN AND ITS MUTANTS

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division

Application deficiencies found during scanning:

☐ Page(s)_____ of _____ were not present for scanning. (Document title)

☐ Page(s)_____ of _____ were not present for scanning. (Document title)

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